Segurança e biodisponibilidade de suplementos alimentares

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RESUMO

O consumo de suplementos alimentares está generalizado e em crescimento constante. Tem como função primordial a complementação, e não substituição, da dieta normal, garantindo a ingestão adequada de nutrientes. No entanto, estes produtos têm sido comercializados com outros objetivos, nomeadamente a prevenção da doença, a manutenção e melhoria da saúde, a melhoria do desempenho físico, ou a manutenção ou obtenção do peso corporal desejado.

A regulamentação dos suplementos alimentares tem sido alvo de harmonização na União Europeia (UE) a fim de garantir a segurança dos consumidores e a livre circulação de produtos entre países membros. Os suplementos na UE são regulamentados por diretivas, as quais especificam os ingredientes passíveis de utilização na sua preparação. No entanto, estas listas positivas estão apenas definidas para vitaminas e minerais. Para além destes nutrientes, os suplementos alimentares podem ainda conter uma infinidade de outras substâncias com efeito nutricional ou fisiológico que não se encontram diretamente abrangidas pela diretiva, sendo regidas pela legislação nacional de cada Estado-Membro.

Adicionalmente, os produtos alimentares não são sujeitos a um controlo rigoroso semelhante ao dos medicamentos, sendo da responsabilidade do operador económico garantir a conformidade com os requisitos da legislação vigente.

A falta de conformidade com os valores rotulados tem sido reportada para vários produtos disponíveis no mercado. Torna-se, assim, necessário conhecer os produtos que se encontram no mercado e os seus ingredientes, garantir a segurança das dosagens recomendadas, e avaliar a real biodisponibilidade dos diversos compostos.

O objetivo deste trabalho foi o de monitorizar os teores de compostos bioativos em determinados grupos de suplementos alimentares, procurando obter/melhorar a informação disponível. Esta informação poderá ser útil para o consumidor que assim pode ter uma noção dos riscos e benefícios do consumo de suplementos alimentares, mas também para os profissionais de saúde para que, com maior segurança, possam aconselhar a ingestão de suplementos alimentares.

De entre os diferentes suplementos alimentares disponíveis no mercado, vários produtos (n=82) foram selecionados e analisados: suplementos à base de extratos vegetais, multivitaminas, minerais, ácidos gordos ómega-3 e fitoestrogénios (n=82). Foram avaliados vários compostos bioativos (compostos fenólicos totais, flavonoides

totais, isoflavonas, ácido ascórbico, ácidos gordos ómega-3, vitamina E e selénio). Para tal, otimizaram-se e/ou aplicaram-se várias metodologias analíticas, nomeadamente técnicas espectrofotométricas, métodos cromatográficos acoplados a diferentes tipos de detetores (HPLC-DAD, HPLC-DAD-FL e GC-FID) e absorção atómica (HR-CS ETAAS). Foi ainda estudada a atividade anti-radicalar de alguns suplementos e a biodisponibilidade de isoflavonas em suplementos contendo fitoestrogénios utilizando a linha celular Caco-2.

Os resultados mostraram que a rotulagem nem sempre descreve a composição do produto que contém.

Como esperado, os resultados referentes à atividade antioxidante dos diferentes suplementos apresentaram uma grande variabilidade, o que reflete a sua diversificada composição e concentração. Em geral, os resultados obtidos confirmam o potencial antioxidante dos suplementos avaliados. Constatou-se que a associação de diferentes antioxidantes pode oferecer algumas vantagens adicionais. Relativamente aos suplementos alimentares à base de selénio, os resultados obtidos experimentalmente mostraram-se concordantes com os níveis médios reclamados nos rótulos dos produtos. Para a validação do método foi utilizado um material de referência certificado. Na análise dos suplementos alimentares enriquecidos com ácidos gordos ómega-3, verificaram-se diferenças significativas para os teores de óleo, EPA, DHA, ALA e vitamina E rotulados para a maioria das amostras estudadas. Finalmente, na análise de suplementos com fitoestrogénios na sua composição, verificaram-se diferenças significativas nas concentrações de isoflavonas determinadas e os teores rotulados. A biodisponibilidade das isoflavonas presentes nos extratos parece depender da concentração de isoflavonas presente e da matriz do produto.

Palavras-chave: suplementos alimentares; controlo de qualidade; antioxidantes; selénio; ómega-3; isoflavonas.

ABSTRACT

The consumption of food supplements is widespread and growing. The main function is to complement, and not replace, the normal diet, ensuring an adequate intake of nutrients. Food supplements have been used for different purposes, in particular for disease prevention, maintaining and improving health, improving physical performance, and maintenance or acquisition of desired body weight.

The regulation of dietary supplements has been object of harmonization in the European Union (EU) in order to ensure consumer safety and free movement of goods between member countries. The supplements are regulated by EU directives, which specify the ingredients that may be used in its preparation. However, these positive lists are only defined for vitamins and minerals. In addition to these nutrients, food supplements may contain a plethora of other substances with nutritional or physiological effect that are not directly covered by this legislation, and are governed by the national law of each Member-State.

Additionally, the regulations for dietary supplements are different and less strict than those for medicines, being the economic operator responsible to ensure compliance with the requirements of current legislation.

The lack of compliance with the labelled values has been reported for several products available in the market. Therefore, it becomes necessary to know those products and their ingredients, ensuring the safety of the recommended dosages, and evaluate the actual bioavailability of the different compounds.

The aim of this study was to monitor the content of bioactive compounds present in selected groups of food supplements, in order to improve the available information about them, contributing for consumers elucidation about the risks and benefits of food supplements consumption, as well as health professionals that, with greater security, can advise the intake of food supplements.

Among the different dietary supplements available in the market, several products (n=82) were selected and analyzed: supplements containing plant extracts, multivitamins, minerals, omega-3 fatty acids and phytoestrogens. Several bioactive compounds (total phenolics, total flavonoids, isoflavones, ascorbic acid, omega-3 fatty acids, vitamin E and selenium) were evaluated. To achieve that, different analytical methodologies were optimized and/or applied: spectrophotometric techniques, chromatographic methods coupled to different detectors (HPLC -DAD, HPLC -DAD -FL and GC -FID) and atomic

absorption (HR- CS ETAAS). The anti-radical activity of some samples and the bioavailability of isoflavones in supplements containing phytoestrogens using the Caco- 2 cell line were also evaluated.

The results showed that the label does not always describe the real composition of the product.

As expected, the results regarding the antioxidant activity of different supplements showed a large variability, which reflects their diverse composition and concentration. Generally, the results confirmed the antioxidant potential of the supplements analysed. It was found that the use of mixtures of antioxidants may offer some additional advantages. Regarding selenium-based food supplements, the experimental results showed good agreement with the average levels claimed on product labels. The accuracy of the method was checked with a certified reference material. In the analysis of dietary supplements enriched with omega-3, there were significant differences in the levels of oil, EPA, DHA, ALA and vitamin E labelled for most of the samples. Finally, the analysis of supplements with phytoestrogens in its composition, showed significant differences in the concentrations of isoflavones and the labelled contents. The bioavailability of isoflavones present in supplements extracts seems to depend on the isoflavone concentration and the product matrix.

Keywords: food supplements; quality control; antioxidants; selenium; omega-3 fatty acids; isoflavones

LISTA DE PUBLICAÇÕES

O trabalho de investigação apresentado nesta tese foi desenvolvido na Faculdade de Farmácia da Universidade do Porto, de 2010 a 2013, tendo resultado nos seguintes trabalhos já publicados ou submetidos para publicação:

Capítulo de Livro

Almeida, I., Fernandes, T.J.R., Guimarães, B.M.R., Oliveira, M.B.P.P. 2014. Omega-3 dietary intake: review on supplementation, health benefits and resources sustainability In: Khan, W. (Ed.) Omega-3 Fatty Acids: Chemistry, Dietary Sources and Health Effects. Nova Science Publishers, Inc., New York, pp. 191-211. ISBN: 978-1-62948-524-9.

Publicações em jornais com arbitragem científica

Dietary antioxidant supplements: Benefits of their combined use.
Almeida, I.M.C., Barreira, J.C.M., Oliveira, M.B.P.P., Ferreira, I.C.F.R.
Food and Chemical Toxicology, **2011**, 49, 3232–3237.

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Costa, A.S.G., Nunes, M.A., Almeida, I.M.C., Carvalho, M.R., Barroso, M.F., Alves, R.C., Oliveira, M.B.P.P.
LWT - Food Science and Technology, **2012**, 49, 324-328.

Label compliance in omega-3 dietary supplements: oil, fatty acids and vitamin E contents analysis. Almeida, I., Fernandes, T.J.R., Oliveira, M.B.P.P.
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Total selenium content of food supplements by microwave digestion and HR-CS ETAAS. Label accuracy evaluation.
Almeida, I.M.C., Oliva-Teles, M.T., Delerue-Matos, C., Oliveira, M.B.P.P.
Journal of Pharmaceutical and Biomedical Analysis (submetido)

Food supplements intended for menopause symptoms relief: isoflavones levels and bioavailability using Caco-2 cell model.
Almeida, I.M.C., Rodrigues, F., Sarmento, B., Alves, R. C., Oliveira, M.B.P.P.
Food Chemistry (submetido)

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Antioxidant potential of individual or combined dietary supplements.
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5th FIP Pharmaceutical Sciences World Congress Melbourne, Australia 13-16 April 2014. (aceite)

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Determination of selenium in dietary supplements by HR-CS ETAAS
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Chemical Reactions in Foods VII, 14-16 November 2012, Prague, Czech Republic.

Antioxidant potential of individual or combined dietary supplements.

Ivone M.C. Almeida, João C.M. Barreira, M. Beatriz P.P. Oliveira, Isabel C.F.R. Ferreira. III Congress of the Portuguese Society of Pharmaceutical Sciences IX Spanish-Portuguese Conference on Controlled Drug Delivery "New Trends in Pharmaceutical Sciences" Oporto, 2011. Pre-Congress Symposium "New Regulatory Developments in Pharmacokinetic Assessment, 13-15 Outubro 2011, Lisboa, Portugal.

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ÍNDICE

ABREVIATURAS

ADN ácido desoxirribonucleico

AHA American Heart Association

ALA ácido α-linolénico

ASAE Autoridade de Segurança Alimentar e Económica

DCV doença cardiovascular

DAD deteção por díodos

DAD/FLD detetores de díodos e fluorescência

DHA ácido docosahexaenóico

DPPH 1,1-diphenyl-2-picrylhydrazyl free radical

CE Comunidade Europeia

AESA/EFSA Autoridade Europeia para a Segurança Alimentar/ *European Food Safety Authority*

EPA ácido eicosapentaenóico

AG ácidos gordos

FAME ésteres metílicos de ácidos gordos/ *fatty acid methyl esters*

FD deteção de fluorescência

FID deteção de ionização de chama/ flame ionization detection

GC cromatografia gasosa

HPLC cromatografia líquida de alta precisão/ *high performance liquid chromatography*

HR-CS ETAAS espectrofotometria de absorção atómica de alta resolução de fonte contínua com atomização eletrotérmica

LOD limite de deteção

LOQ limite de quantificação

MSPD dispersão da matriz em fase sólida/ *matrix solid phase dispersion*

MUFA ácidos gordos monoinsaturados

n-3 ómega-3

DDR dose diária recomendada

ROS espécies reativas de oxigénio

RNS espécies reativas de azoto

PUFA ácidos gordos polinsaturados

UE União Europeia

UV / VIS ultravioleta-visível

λ comprimento de onda

1. INTRODUÇÃO

1. INTRODUÇÃO

Um regime alimentar adequado, por definição, previne deficiências nutricionais, fornecendo nutrientes e energia suficientes para o crescimento e reprodução (1). A influência da dieta e do estado nutricional na etiologia de várias doenças crónicas está hoje bem estabelecida, não só a nível institucional, por organizações de saúde e entidades reguladoras, mas também por uma parcela crescente da população (2) que procura ter uma dieta mais saudável.

Nas últimas décadas, as sociedades têm modificado os seus padrões de vida, e os consumidores estão cada vez mais interessados em assumir um papel ativo na sua saúde e bem-estar (3). A indústria alimentar e farmacêutica, atenta a estas tendências, vem desenvolvendo e lançando no mercado um número crescente de produtos com propriedades promotoras da saúde, incluindo alimentos funcionais, nutracêuticos e suplementos alimentares.

A ingestão de suplementos alimentares visa complementar a dieta normal, garantindo a ingestão de nutrientes em quantidade e qualidade adequadas. No entanto, estes produtos têm sido utilizados com diferentes objetivos, nomeadamente a prevenção da doença, a manutenção e melhoria da saúde, a melhoria do desempenho físico, ou a obtenção e/ou a manutenção do peso corporal desejado. Cada vez mais pessoas recorrem a estes produtos em todo o mundo, tornando a avaliação da qualidade e segurança destes produtos uma questão premente para os governos e para a comunidade científica.

A regulamentação dos suplementos alimentares tem sido alvo de harmonização na União Europeia (UE) a fim de garantir a segurança dos consumidores e a livre circulação de produtos entre países membros. Os suplementos na UE são regulamentados por diretivas e as suas respetivas alterações, as quais especificam os ingredientes passíveis de utilização na sua preparação. No entanto, estas listas positivas estão apenas definidas para vitaminas e minerais. Para além destes nutrientes, os suplementos alimentares podem ainda conter uma infinidade de outras substâncias com efeito nutricional ou fisiológico, incluindo aminoácidos, ácidos gordos essenciais, fibras, enzimas, várias plantas e extratos de plantas, probióticos e prebióticos, entre outros, que não se encontram regulamentados. Adicionalmente, estes produtos não são sujeitos a um controlo rigoroso, semelhante aos medicamentos, sendo da responsabilidade do operador económico garantir a conformidade com os requisitos da legislação vigente.

O número crescente e diversificado de suplementos alimentares disponíveis para consumo requer um controlo da qualidade destes produtos ao nível dos seus ingredientes e dosagens recomendadas, e da real biodisponibilidade dos diversos compostos presentes, por forma a demonstrar o seu efeito benéfico.

1.1 Suplementos Alimentares

1.1.1 Enquadramento Legal e Definição de Suplemento Alimentar

Até 2002, os suplementos alimentares na UE estavam sujeitos às regulamentações nacionais, que variavam consideravelmente em toda a UE, criando obstáculos à sua livre comercialização entre os Estados-Membros.

A 10 de junho de 2002, foi publicada a Diretiva nº 2002/46/CE do Parlamento Europeu e do Conselho, referente à regulamentação dos suplementos alimentares, e transposta em Portugal através do Decreto-Lei n º 136 /2003 de 28 de junho de 2003 (4, 5). Esta Diretiva tem como objetivo principal harmonizar a legislação relativa aos suplementos vitamínicos e minerais em toda a Europa, e garantir que os produtos disponibilizados no mercado são seguros, claramente rotulados, e não reivindicam propriedades medicinais, de forma a possibilitar uma escolha informada por parte dos consumidores.

Os suplementos alimentares são definidos no Artigo 2.º da Diretiva como "*géneros alimentícios que se destinam a complementar e ou suplementar o regime alimentar normal e que constituem fontes concentradas de determinadas substâncias nutrientes ou outras com efeito nutricional ou fisiológico, estremes ou combinadas, comercializadas em forma doseada, tais como cápsulas, pastilhas, comprimidos, pílulas e outras formas semelhantes, saquetas de pó, ampolas de líquido, frascos com conta-gotas e outras formas similares de líquidos ou pós que se destinam a ser tomados em unidades medidas de quantidade reduzida, onde "nutrientes" são as seguintes substâncias: i) vitaminas, ii) minerais*".

Na sua composição, além de vitaminas e sais minerais, podem ainda conter outras substâncias com efeito nutricional ou fisiológico, nomeadamente: aminoácidos, ácidos gordos essenciais, fibras e várias plantas e extratos de ervas.

A lista de vitaminas e sais minerais permitidos para utilização nos suplementos alimentares, assim como a forma química em que se podem encontrar, estão presentes no Anexo I e no Anexo II da Diretiva. Esta lista foi posteriormente alterada pela Diretiva 2006/37/CE da Comissão (transposta para Portugal pelo Decreto-Lei n.º 296/2007, de 22 de Agosto) (6), o Regulamento (CE) N.º 1170/2009, o Regulamento (UE) N.º 1161/2011 e o Regulamento (UE) N.º 119/2014 para incluir novas substâncias (7-9).

A Diretiva contempla cinco aspetos principais relativos à regulamentação dos suplementos vitamínicos e minerais: especificar as vitaminas e minerais permitidos e as suas fontes químicas; fixar as quantidades máximas e mínimas de vitaminas e minerais permitidas em suplementos; definir quais as regras para a rotulagem; proibir os fabricantes/distribuidores de suplementos de fazer quaisquer alegações terapêuticas nos seus produtos; proibir os Estados-Membros de restringir o comércio de produtos quando estes se encontram conformes com a diretiva.

Na UE, a Autoridade Europeia para a Segurança dos Alimentos (AESA) é o organismo responsável pela implementação e monitorização destas substâncias, enquanto em Portugal esta função cabe ao Gabinete de Planeamento e Políticas (GPP), sob a tutela do Ministério da Agricultura e do Mar (6, 10). Para colocar um suplemento alimentar no mercado, o operador económico tem primeiro de notificar o GPP, enviando o rótulo para aprovação. De acordo com os princípios consagrados no Regulamento (CE) N.º 178/2002 de 28 de Janeiro, é da responsabilidade do operador económico garantir a conformidade com os requisitos da legislação vigente (11). A Autoridade de Segurança Alimentar e Económica (ASAE) é a autoridade nacional competente para a fiscalização, avaliação e comunicação dos riscos na cadeia alimentar, que será sempre feita retrospetivamente (não se trata de uma condição prévia à sua comercialização).

1.1.2 Uso de Suplementos Alimentares

O crescente interesse e uso de suplementos alimentares pela população em geral, tornou a indústria de suplementos num dos negócios mais lucrativos do mercado da saúde atualmente, tendo sido avaliado globalmente em cerca de 84 mil milhões de dólares em 2011 (12).

Os diversos estudos realizados para avaliar os hábitos de consumo de suplementos alimentares em diferentes populações têm revelado algumas tendências, nomeadamente no que se refere a atitudes e estilo de vida, além de outros aspetos sociodemográficos

como o género, a faixa etária e a escolaridade. Verifica-se uma maior prevalência do uso de suplementos entre as mulheres (13-16) em indivíduos de idade mais avançada (15-18), em indivíduos com maior escolaridade e/ou estatuto socioeconómico mais elevado (15, 16, 19) nos atletas (20, 21), e em indivíduos com menor índice de massa corporal (IMC) (16, 22, 23). Vários estudos demonstraram também um elevado uso de suplementos em grupos de indivíduos portadores de determinadas patologias (24, 25).

O principal motivo apontado para o uso de suplementos alimentares é a promoção da saúde ou a prevenção de doenças (13, 26). A maioria dos estudos têm mostrado que os utilizadores de suplementos tendem a apresentar um estilo de vida mais saudável (16, 17, 22, 27, 28) uma menor incidência de diabetes (29) e de doenças cardiovasculares (30).

1.1.2.1 Prevalência do uso de Suplementos Alimentares na população portuguesa

Em Portugal, o consumo de suplementos alimentares tem vindo a aumentar nas últimas décadas (13), seguindo padrões de consumo semelhantes aos internacionais. Entre 2008 e 2012, como resultado da crise económica, observou-se uma contração do mercado dos suplementos alimentares, tendência observada igualmente em outros países da UE (12). Dados de consumo recentes mostram que em 2013, 12,7% dos residentes no continente referiram ter consumido suplementos nos últimos 12 meses (31). Existem alguns estudos de prevalência de uso de suplementos alimentares em Portugal visando populações específicas, tais como estudantes (32, 33), atletas (34), frequentadores de ginásios (35), e grávidas (36), mas também classes de suplementos alimentares específicas (37). Em 2006, foi realizado um estudo transversal da população portuguesa, encomendado pela ASAE, com o objetivo de caracterizar o consumo de suplementos alimentares em Portugal (13). De acordo com este estudo, 65 % da população referiu ser consumidora de suplementos alimentares, com 72% da população adulta a referir a sua utilização no ano anterior. Os suplementos mais consumidos foram as vitaminas (65 %) e minerais (52 %), seguido de suplementos à base de plantas (38%), e suplementos para fornecer “energia” (34 %). O uso de suplementos foi mais prevalente entre as mulheres (63%). As motivações apontadas para tomar suplementos relacionam-se com a fadiga e dificuldades de concentração (26%), fortalecimento e prevenção de doença (24%), saúde (22%), estética (10%), entre outros (17%) (13). A maioria da população (47%) toma suplementos por períodos irregulares e limitados, apesar de 29% os consumir ao longo do ano e 24% serem consumidores sazonais (13). Em Portugal, os suplementos alimentares são distribuídos através de vários pontos de venda, incluindo

farmácias e parafarmácias, ervanárias, lojas de produtos naturais, supermercados e *online*.

1.2 Suplementos Alimentares em estudo na presente tese

1.2.1 Suplementos com ação Antioxidante

De entre os diferentes suplementos alimentares disponíveis no mercado optou-se por avaliar suplementos à base de extratos vegetais, multivitaminas, minerais, ácidos gordos ómega-3 e fitoestrogénios, suplementos com elevado consumo no mercado.

1.2.1.1 Radicais livres

Um radical livre é definido como qualquer espécie que possui um ou mais eletrões desemparelhados na orbital exterior (38). Nos sistemas vivos, os radicais livres são produzidos principalmente durante o metabolismo aeróbio normal, na sua maioria sob a forma de espécies reativas de oxigénio (ROS) e de azoto (RNS) (39). A sua formação ocorre maioritariamente na mitocôndria, e, quando em níveis fisiológicos, funcionam como mediadoras de importantes cascatas de sinalização intracelular em numerosos processos metabólicos, tais como: mecanismos de defesa e regulação da resposta imunitária, a produção de energia, e a transcrição de genes, entre outros (40). Durante o funcionamento normal da célula, a maior parte destes radicais livres é removida pelas defesas celulares antioxidantes (41). Em determinadas situações, a formação excessiva de espécies reativas ou a diminuição dos níveis de antioxidantes conduzem ao stresse oxidativo, isto é, a um desequilíbrio oxidante/antioxidante (40). Este desequilíbrio tem como consequência o aumento dos danos causados pelas ROS a biomoléculas fundamentais (lípidos, proteínas, enzimas, DNA, e RNA) que, quando não reparados, acabam por prejudicar a função celular, e podem desencadear várias doenças (42). O stresse oxidativo pode resultar da exposição a fatores ambientais (fumo, poluição, radiação ultravioleta, alto teor de gordura na dieta, entre outros) ou de fatores naturais como uma atividade física intensa, ou um processo de inflamação resultante de doença. Esta condição tem sido implicada na iniciação e/ou na progressão de uma variedade de condições crónicas, incluindo as doenças cardiovasculares, diabetes, e vários cancros, entre outras, mas, também, no processo de envelhecimento em geral (38, 40).

1.2.1.2 Antioxidantes

De um modo geral, o termo antioxidante designa uma molécula, um ião, ou um radical relativamente estável que é capaz de retardar ou evitar a oxidação de outras moléculas. Nos sistemas biológicos, a proteção contra danos induzidos pelos radicais livres é fornecida por sistemas de defesa antioxidante complexos, que compreendem antioxidantes endógenos (enzimáticos e não enzimáticos) e exógenos (41, 42). As defesas antioxidantes enzimáticas são em grande número e encontram-se espalhadas por todo o organismo, tanto no meio intracelular como no meio extracelular. Estas defesas incluem a superóxido dismutase (SOD), a catalase (CAT), a glutationa peroxidase (GSH-Px), a glutationa redutase (GSH-R), entre outras (42, 43). Estas enzimas protegem as células aeróbias da ação de diferentes espécies reativas incluindo do anião superóxido ($O_2^{\bullet -}$), do peroxido de hidrogénio (H_2O_2) e de hidroperóxidos orgânicos (ROOH). As defesas antioxidantes enzimáticas apresentam interações sinérgicas, a fim de manter os níveis de radicais livres dentro dos limites fisiológicos. Enquanto a SOD e a CAT atuam principalmente em regiões hidrofílicas, a GSH-Px protege regiões hidrofóbicas, com especificidade para peróxidos lipídicos (42).

Além das enzimas, as células contêm outros compostos antioxidantes que lhes conferem uma maior proteção contra os radicais livres, os antioxidantes exógenos. Estes provêm principalmente da dieta, sendo a maioria de origem vegetal e designam-se por fitoquímicos. Com efeito, numerosos estudos epidemiológicos e clínicos verificaram uma relação inversa entre a ingestão de uma dieta rica em alimentos de origem vegetal (fruta, vegetais, e grãos) e a incidência de doenças crónicas, incluindo diabetes, aterosclerose, artrite reumatóide, doenças neurodegenerativas e coronárias e cancro (29, 44-48). Os efeitos benéficos associados a estas dietas têm sido atribuídos, em parte, à complexa mistura de compostos antioxidantes presentes.

Com a hipótese da associação do stresse oxidativo à patogénese de diversas doenças, surgiram as expectativas de que os antioxidantes poderiam ser utilizados para reduzir a mortalidade e/ou morbilidade de pessoas suplementadas com estes compostos, pela neutralização dos radicais livres nocivos (49).

O crescimento contínuo do mercado de antioxidantes e o interesse dos consumidores por estes produtos parece resultar da noção generalizada de que estes compostos podem prevenir ou tratar várias doenças, que crê-se serem causadas ou promovidas pelo stresse oxidativo (39, 50).

Por estas razões, o desenvolvimento de produtos contendo antioxidantes continua a merecer o investimento de muito tempo, dinheiro e esforço pelos sectores alimentar e biotecnológico, sendo lançados anualmente centenas de produtos no mercado alegando "poder antioxidante".

No entanto, a abordagem nutricional à terapia antioxidante está ainda pouco compreendida, devido à complexidade dos diferentes fatores que relacionam as doenças degenerativas, a dieta e a oxidação. Os resultados da maior parte dos estudos in vitro e in vivo realizados para avaliar os efeitos dos antioxidantes dietéticos em sistemas biológicos são de difícil interpretação devido às diferentes metodologias que têm sido utilizadas para medir a oxidação e a suscetibilidade oxidativa das diferentes biomoléculas. A ausência de biomarcadores do stresse oxidativo fiáveis contribui também para esta dificuldade.

Os suplementos alimentares antioxidantes consistem em formulações contendo um ou mais compostos com efeito antioxidante, de origem natural ou sintética, e são apresentados sob uma grande variedade de formas, incluindo comprimidos, pílulas, cápsulas, pós, bebidas e barras. As formulações antioxidantes podem utilizar uma infinidade de ingredientes, incluindo vitaminas antioxidantes (como os tocoferóis e o ácido, ascórbico); outros compostos bioativos isolados de origem vegetal como carotenoides (e.g. α-caroteno, β-caroteno, luteína, zeaxantina, astaxantina e licopeno); compostos fenólicos, flavonoides (e.g. flavonóis, antocianidinas e isoflavonoides), compostos azotados (e.g. aminoácidos) e compostos organossulforados (e.g. isotiocianatos), bem como, extratos de plantas e algas, frutas e legumes concentrados, enzimas, minerais (selénio, zinco, manganês), polissacarídeos, entre outros.

1.2.1.3 Suplementos com Selénio

O selénio é um micronutriente essencial para o crescimento, desenvolvimento e metabolismo normal de animais e seres humanos (51). Constitui uma parte integrante de selenoproteínas, incluindo a glutationa peroxidase (GSHPx), uma enzima antioxidante que protege as membranas celulares contra danos dos radicais livres, as iodotironinas desiodases, envolvidas no metabolismo da hormona da tiróide, e a tioredoxina redutase, que, em conjunto com o composto de tiorredoxina, participa na regeneração de vários antioxidantes quando nas suas formas oxidadas, e na regulação do crescimento e viabilidade celular (52).

O selénio é introduzido na cadeia alimentar através das plantas, e de produtos que delas derivam, que assimilam os compostos de selénio presentes no solo. Assim, o teor de selénio nos alimentos vai depender da sua origem geográfica e, consequentemente, a ingestão de selénio por pessoas e animais vai variar entre países e regiões (53). As principais fontes alimentares de selénio para o homem são os mariscos e vísceras e, em menores quantidades, os cereais, grãos e produtos lácteos (54).

Atualmente, a Dose Diária Recomendada (DDR) para o selénio é de 55 µg/dia para homens e mulheres adultos e saudáveis, que corresponde à quantidade estimada necessária para a maximização da atividade da GSHPx no plasma (55). No entanto, existem evidências clínicas de que a ingestão de selénio em doses superiores à DDR (200-300 µg por dia) pode prevenir certos tipos de cancro (56, 57), doenças cardiovasculares (58), e melhorar a resposta imunológica, bem como, a fertilidade masculina (59, 60). Apesar destes indicadores, a maioria dos países europeus, incluindo Portugal, regista uma deficiente ingestão de selénio por parte da população (61, 62). A suplementação poderá constituir uma opção para garantir os níveis nutricionais recomendados ou, utilizada com o objetivo da prevenção de algumas doenças e do envelhecimento.

Nas últimas décadas, o selénio tem atraído muito interesse como agente antitumoral devido ao seu papel como antioxidante (63, 64). A constatação da presença de níveis baixos de selénio nos alimentos, dependendo da região, e o reconhecimento do seu potencial na saúde, fez com que os suplementos alimentares enriquecidos com selénio ganhassem interesse e popularidade. Apesar de ser um mineral essencial, é tóxico se ingerido em excesso, estando a dose máxima admissível fixada em 300-400 µg/dia (55, 65). A intoxicação por selénio é chamada selenose e os sintomas incluem um odor a alho na respiração, distúrbios gastrointestinais, perda de cabelo, descamação das unhas, danos neurológicos e fadiga (55).

Os suplementos alimentares disponíveis no mercado podem conter o selénio estreme, ou combinado em multivitamínicos/multiminerais, sob diferentes formas químicas incluindo selenometionina e leveduras enriquecidas com selénio, selenocisteína, selenito de sódio, selenato de sódio, e selenito de hidrogénio (54, 66). O metabolismo do selénio é determinado parcialmente pela sua forma química e a fonte alimentar. A selenometionina é geralmente, considerada a melhor forma de selénio para ser absorvida e armazenada no corpo humano (67).

1.2.2 Suplementos Alimentares com ácidos gordos ómega-3

Entre os suplementos alimentares que têm recebido um interesse crescente nos últimos anos encontram-se os ácidos gordos polinsaturados (PUFA) e, em particular, os ómega-3 (*n*-3).

A nomenclatura dos PUFA das séries *n*-3 e *n*-6 refere-se à localização da primeira ligação insaturada a partir do extremo metilo (*n*) da cadeia carbonada. A primeira ligação dupla está localizada no carbono 3, para os ácidos gordos *n*-3, e no carbono 6, para os *n*-6. O ácido α-linolénico (18:3) e o ácido linoleico (18:2) são os precursores dos ácidos gordos das séries *n*-3 e *n*-6, respetivamente.

O organismo humano não tem a capacidade de inserir uma ligação dupla antes do carbono 9 na cadeia carbonada do ácido gordo, nem de biossintetizar ácido α-linolénico ou ácido linoleico, e por isso, estes ácidos gordos essenciais devem ser adquiridos através de fontes alimentares. A capacidade do corpo humano para produzir os ácidos gordos polinsaturados da série omega-3 de cadeia longa (*n*-3 LC PUFA), especialmente os ácidos eicosapentaenóico (20:5 *n*-3, EPA) e docosahexaenóico (22:6 *n*-3, DHA), a partir dos seus precursores é limitada, o que torna a sua ingestão vital.

As melhores fontes naturais de PUFA *n*-3 são os peixes gordos, salmão, sardinha e cavala, algas, e vários óleos vegetais edíveis.

Estudos epidemiológicos e clínicos têm demonstrado que o consumo regular de peixe e ácidos gordos ómega-3 diminui o risco de doença cardiovascular (DCV) (68). Com base nessas evidências, a maioria dos grupos e organizações internacionais recomendam à população em geral, uma ingestão diária de pelo menos 250 mg de *n*-3 de LCPUFA (principalmente EPA e DHA), de preferência através do consumo de peixes gordos (1-2 porções por semana) (69-71). Para os pacientes com DCV, a American Heart Association (AHA) recomenda a ingestão de cerca de 1 g/dia de EPA e DHA, de preferência pela ingestão de peixes gordos, embora a suplementação possa ser uma alternativa (69). Para fins de redução dos níveis de triglicéridos em pacientes com hipertrigliceridemia, é recomendada a ingestão de 2-4 g/dia de EPA e DHA, que deverá ser fornecida sob a supervisão de um médico (69, 71).

Apesar da sensibilização dos consumidores para os benefícios na saúde da ingestão de n-3 PUFA, na maioria dos países ocidentais, incluindo Portugal, os níveis de ingestão estimados são menores do que o recomendado atualmente (72, 73). Existem vários fatores que podem contribuir para a baixa ingestão de peixe e/ou produtos do mar

nomeadamente, preferências alimentares, preço, preocupações ambientais, razões éticas, e as dificuldades na preparação (74). Nestes casos, os suplementos alimentares podem oferecer uma alternativa eficaz para aumentar a ingestão de *n*-3 PUFA.

Os estudos sugerem que os seres humanos evoluíram com uma dieta contendo uma proporção de ácidos gordos essenciais *n*-6 e *n*-3 de cerca de 1-2:1 (75). As rápidas mudanças na dieta ocorridas a partir do século XX resultaram num aumento acentuado do consumo de gorduras totais, saturadas e de ácidos gordos da série n-6 e, simultaneamente, numa redução da ingestão de ácidos gordos *n*-3. A ingestão de teores elevados de *n*-6 PUFA, característica da dieta ocidental, pode aumentar a síntese de eicosanoides pró-inflamatórios, dando origem a um estado fisiológico promotor de doenças cardiovasculares (DCV) e de cancro. Pelo contrário, um maior consumo de *n*-3 LCPUFA está associado a uma maior proteção contra a inflamação (75).

Os papéis opostos de *n*-3 e *n*- 6 PUFAs na regulação do processo inflamatório sugerem a importância da relação *n*-6/*n*-3 no desenvolvimento e na gravidade das doenças de natureza inflamatória. Isto é de particular importância quando se considera a profusão de ácidos gordos n-6 na dieta atual e na falta de *n*-3 (75). No entanto, é necessária uma maior evidência experimental, a fim de se recomendar um rácio específico dos ácidos gordos *n*-6 e *n*-3 (71, 76).

O mercado oferece uma enorme variedade de produtos contendo *n*-3 PUFA, obtidos de óleos de peixe, de krill, de algas e de linhaça, ou misturas de óleos. Os *n*-3 PUFA em suplementos alimentares podem apresentar-se em várias formas, incluindo cápsulas moles, óleos e emulsões, e estão disponíveis a partir de vários canais: supermercados, lojas de saúde, ervanárias, na internet, e farmácias.

Os suplementos de *n*-3 PUFA são geralmente bem tolerados, tendo-se observado alguns efeitos secundários ligeiros. Os efeitos adversos mais comuns incluem a eructação e um gosto a peixe, desconforto gastrointestinal e náuseas, e intolerância às cápsulas (77, 78).

De uma forma geral, os suplementos de óleo de peixe são considerados seguros e livres de teores detetáveis de mercúrio (79). Apresentam, em geral, níveis reduzidos de bifenilos policlorados (PCB) (80, 81). Assim, os consumidores preocupados com a possibilidade da presença de contaminantes ambientais no peixe, podem optar pela suplementação.

Estes produtos alegam manter as articulações e músculos saudáveis, a saúde cardiovascular, o desenvolvimento e funcionamento do cérebro, entre outros efeitos benéficos.

1.2.3 Suplementos Alimentares com Fitoestrogénios

As isoflavonas são uma classe de fitoestrogénios, compostos derivados de plantas com atividade estrogénica. A sua analogia estrutural com 17-β-estradiol confere-lhes efeitos hormonais, incluindo a capacidade de se ligar a recetores de estrogénio e de modular processos dependentes de hormonas (82). As isoflavonas têm uma distribuição restrita na natureza, ocorrendo principalmente em plantas da família Fabaceae, incluindo a soja, lentilhas, grão de bico, alfafa, trevo vermelho, e kudzu (83). A soja e os produtos à base de soja são a única fonte conhecida de quantidades significativas de isoflavonas na dieta humana (83).

Estudos epidemiológicos têm relacionado o consumo de dietas ricas em isoflavonas, com a menor incidência dos principais cancros hormono-dependentes (84, 85), de doenças cardiovasculares (86), osteoporose, e da sintomatologia associada com a menopausa (87).

Atualmente estão disponíveis vários suplementos alimentares com isoflavonas na sua composição, visando particularmente as mulheres na menopausa. Estes produtos têm sido referenciados como uma alternativa terapêutica para a sintomatologia da pós-menopausa em algumas situações de contra-indicação para a terapêutica hormonal de substituição (THS), como eventuais riscos associados e efeitos adversos da THS (88, 89). Os suplementos alimentares utilizados na menopausa frequentemente contêm extratos de soja, trevo vermelho, e kudzu, estremes ou em formulações multi-ingredientes com minerais, vitaminas, outros extratos vegetais, ácidos gordos ómega-3, 6 e 9, entre outros.

As três principais isoflavonas da soja (*Glycine max* (L.) Merril) são a daidzeína, a genisteína e a gliciteína, que ocorrem principalmente como glicosídeos, acetilglicosídeos, e malonilglicosídeos. Nos derivados de soja, incluindo os suplementos alimentares, os glicosídeos e as agliconas são os componentes principais (90). O trevo vermelho (*Trifolium pratense* L.) contém numerosas isoflavonas, sendo a biochanina A e a formononetina e seus derivados os principais isoflavonóides presentes (91, 92). Outras plantas usadas pelo seu efeito estrogénico incluem diversas espécies do género Pueraria spp., utilizadas tradicionalmente na medicina popular asiática para atenuar os sintomas da menopausa. Vários isoflavonóides foram identificados nos tubérculos destas plantas incluindo daidzina, puerarina, daidzeína, e genisteína (93).

As isoflavonas, como a maioria dos polifenóis, encontram-se nas plantas, principalmente como glicosídeos e ésteres de glicosídeos (94). Após a ingestão estes são

metabolizados pela flora do trato gastrointestinal, libertando as suas agliconas, os constituintes verdadeiramente bioativos (95, 96).

Apesar de alguns estudos terem indicado, em alguns casos, a existência de contraindicações ao uso de isoflavonas (97), estudos epidemiológicos não têm observado efeitos adversos associados ao seu consumo. No entanto, uma vez que a atividade estrogénica destas moléculas tem sido comparada ao fármaco tamoxifeno, deverá ter-se em conta que o seu uso também pode aumentar o risco de se desenvolver cancro do endométrio (98).

1.3 OBJETIVOS E ORGANIZAÇÃO GERAL DA TESE

Nas últimas três décadas, observou-se um aumento acentuado e generalizado no consumo de suplementos alimentares. A promoção do bem-estar e a prevenção de doenças crónicas, o fortalecimento do sistema imunitário e a melhoria do desempenho desportivo ou da imagem corporal, estão entre os motivos geralmente apontados para a suplementação. Em Portugal, o seu consumo é crescente, acompanhando as tendências verificadas em outros países. Estes produtos encontram-se disponíveis numa grande variedade de formulações e são vendidos através de múltiplos canais, muitas vezes sem o apoio de um profissional. Os suplementos alimentares são considerados pelos consumidores como "naturais" e "seguros", em contraste com os medicamentos convencionais. No entanto, são cada vez mais frequentes as situações em que são postas em causa a qualidade, a segurança e a eficácia destes produtos, surgindo na bibliografia a descrição de efeitos adversos e algumas não conformidades.

A regulamentação dos suplementos alimentares tem sido alvo de harmonização na União Europeia (UE), especialmente para vitaminas e minerais. O fabricante é o único responsável pela segurança/qualidade dos seus produtos, o que pode facilitar a ocorrência de fraude, adulteração, contaminação e a não conformidade relativamente às quantidades rotuladas. Vários estudos têm realçado a escassez de informação nos rótulos sobre ingredientes, concentrações, doses ou efeitos das substâncias presentes, o que pode comprometer a sua eficácia terapêutica e a segurança dos consumidores. A análise dos componentes que constituem os suplementos alimentares é, assim, uma etapa essencial para assegurar a sua utilização segura pelos consumidores.

Nesse sentido, este trabalho teve os seguintes objetivos gerais:

- ✓ Monitorizar os compostos bioativos e fornecer informações sobre diferentes suplementos alimentares disponíveis no mercado Português.
- ✓ Contribuir para o melhor conhecimento destes produtos, a fim de beneficiar da presença dos compostos químicos bioativos presentes e minimizar os potenciais efeitos adversos na saúde.

Para atingir os objetivos gerais acima enunciados, desenvolveu-se um conjunto de tarefas laboratoriais procurando dar resposta aos seguintes objetivos específicos:

- ✓ Avaliar suplementos alimentares antioxidantes nomeadamente, quantificar alguns compostos bioativos (compostos fenólicos totais, flavonoides totais, ácido ascórbico, selénio) e determinar a atividade anti-radicalar dos

extratos. Avaliar vantagens/desvantagens da toma associada de diferentes produtos, e a conformidade com a rotulagem.

- ✓ Analisar o perfil de ácidos gordos de suplementos à base de óleo de peixe, óleo de krill e/ou óleos vegetais e verificar a conformidade em relação aos valores rotulados relativamente aos teores de óleo, dos ácidos gordos ómega-3, EPA, DHA, ALA e vitamina E.
- ✓ Determinar o perfil de isoflavonas de suplementos à base de fitoestrogénios provenientes de diferentes fontes vegetais, nomeadamente, soja, trevo-vermelho e Thai-kudzu. Verificar a conformidade em relação aos valores rotulados. Determinar a biodisponibilidade das isoflavonas nos extratos de suplementos selecionados, usando a linha celular Caco-2.

A presente tese é constituída por 3 capítulos, que tratam respetivamente a Introdução à temática dos suplementos alimentares, os resultados obtidos e as considerações finais. Por sua vez, o capítulo referente aos resultados é constituído por 3 subcapítulos (artigos científicos). A tese está escrita em português, seguindo a "Regra de Vancouver" para as referências.

No Capítulo 1 apresenta-se uma panorâmica geral sobre os suplementos alimentares, relativamente ao enquadramento legal e ao mercado, focando em particular a realidade portuguesa. É feita uma reflexão sobre o uso de suplementos alimentares e a necessidade de controlo da qualidade desses produtos, focando especificamente os suplementos com efeito antioxidante, os suplementos à base de ácidos gordos ómega-3, e os suplementos à base de isoflavonas. É feita uma descrição dos compostos bioativos característicos de cada classe de suplementos analisados, e são apresentados alguns resultados descritos na literatura.

Segue-se o Capítulo 2, a parte experimental da tese, que é constituída por 3 partes, correspondendo cada uma delas a um grupo de suplementos alimentares: suplementos alimentares com ação antioxidante (2.1), suplementos contendo ácidos gordos ómega 3 (2.2) e suplementos alimentares contendo fitoestrogénios (2.3). Optou-se por manter a formatação original, adaptada ao corpo da tese, com a qual os textos foram submetidos ou publicados, de acordo com as normas específicas de cada revista.

A primeira parte refere-se à avaliação de suplementos antioxidantes. É constituída por 3 artigos relativos à determinação de compostos bioativos presentes neste tipo de suplementos e avaliada a atividade antioxidante dos seus extratos.

A segunda parte é dedicada ao estudo de suplementos alimentares contendo ácidos gordos ómega-3. Deste trabalho resultou um artigo referente à determinação do perfil dos ácidos gordos das amostras, aos teores de compostos bioativos e à comparação dos resultados obtidos com a informação indicada no rótulo.

A terceira parte descreve o trabalho realizado com suplementos alimentares à base de fitoestrogénios nomeadamente, a determinação dos compostos bioativos e a avaliação da biodisponibilidade intestinal das isoflavonas presentes nos extratos destes produtos.

O último capítulo refere-se às "considerações finais", onde é feita uma síntese dos principais resultados obtidos para cada classe de suplementos alimentares. Tece-se um conjunto de reflexões sobre o trabalho realizado, e apresentam-se algumas questões que poderão ser retomadas em futuras investigações realizadas neste domínio.

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2. RESULTADOS

2.1– Suplementos Alimentares com atividade Antioxidante

A. Dietary antioxidant supplements: Benefits of their combined use. *Food and Chemical Toxicology, 2011, 49, 3232–3237*

B. Teas, dietary supplements and fruit juices: A comparative study regarding antioxidant activity and bioactive compounds*. LWT - Food Science and Technology, 2012, 49, 324-328*

C. Total selenium content of food supplements by microwave digestion and HR-CS ETAAS. Label accuracy evaluation. (*submitted*)

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Dietary antioxidant supplements: benefits of their combined use

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Abstract

Several dietary supplements claim medicinal benefits due to their composition in hydrophilic and lipophilic molecules, natural extracts or synthetic compounds with antioxidant properties. In the present work, the antioxidant activity of selected supplements taken in pills, capsules or infusions were studied either individually or combined. Linear discriminant analysis (LDA) was used to categorize the condensed formulations (pills and capsules), infusion bags and combined samples according with their antioxidant activity measured by radical scavenging activity, reducing power and lipid peroxidation inhibition using brain homogenates as models. AAF proved to have the highest antioxidant activity in all the assayed methods, either singly taken or included in mixtures. Furthermore, the mixtures containing this supplement revealed synergistic effects in 92% of the cases. The intake of antioxidant mixtures might provide some additional benefits.

Keywords: Dietary Supplements; Antioxidant activity; Synergistic Effects; Linear Discriminant Analysis.

1. Introduction

In living systems, Reactive Oxygen/Nitrogen Species (ROS/RNS) are produced primarily during normal aerobic metabolism (Halliwell and Gutteridge, 2007). At physiological levels, these intermediates participate in numerous metabolic processes including cell signaling, energy production, gene transcription and immune defense, among others (Seifried et al., 2007). However, decline of antioxidant defense mechanisms or exposure to environmental factors (smoke, pollution, ultraviolet radiation, high-fat diet, etc.) and pathological conditions (chronic infection, inflammation, etc.) can lead to increased ROS/RNS production, resulting in oxidative stress (Valko et al., 2007). Oxidative stress can damage key organic substrates such as DNA, lipids and proteins, compromising cells physiological function (Nordberg and Arnér, 2001). This condition has been associated to the ageing process in general, and to the initiation and progression of a variety of chronic conditions related to it, such as cardiovascular disease and cancer (Valko et al., 2007).

Protection against ROS/RNS-induced damage is provided by complex antioxidant defense systems, comprising endogenous enzymatic and non-enzymatic antioxidants (e.g., superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase) and exogenous antioxidants (e.g., vitamin C, vitamin E, carotenoids and polyphenols), the latter provided mainly by the diet (Young and Woodside, 2001). Indeed, numerous epidemiological and clinical studies have linked high intake of fruits, vegetables, whole grains, and beverages of plant origin, which are rich in antioxidants, with lower incidence and mortality rates of chronic diseases including diabetes, atherosclerosis, rheumatoid arthritis, neurodegenerative and coronary diseases and cancer (Cerhan et al., 2003, de Kok et al., 2010, Esposito et al., 2002, Ford and Mokdad, 2001, Hertog et al., 1993 and Kris-Etherton et al., 2002). These potential physiological benefits of dietary antioxidants have lead, in recent years, to a dramatic growth of the market of functional foods and dietary supplements claiming “antioxidant power”, and to the widespread consumption of these products.

Antioxidant dietary supplements are sold as isolated substances or as mixtures, from natural or synthetic origin, and are presented in a variety of forms including tablets, pills, capsules, powders, drinks and supplement bars. Antioxidant formulations use a plethora of ingredients, including antioxidant vitamins (tocopherols, ascorbic acid), bioactive compounds of plant origin (polyphenols and carotenoids), plant and algae extracts, fruits and vegetables concentrates, enzymes, minerals (selenium, zinc, manganese), polysaccharides, organosulfur compounds, etc.

The antioxidant activity of foodstuffs as well as the purified bioactive compounds to be used in supplement formulations, has been intensely researched (Barreira et al., 2008, Borges et al., 2010, Gorinstein et al., 2011, Müller et al., 2011, Stratil et al., 2007 and Tabart et al., 2009). However, data regarding antioxidant activity of formulations already on the market is scarce. These products are promoted has antioxidant boosters but labels often lack information regarding effective antioxidant capacity values.

Therefore, the present study aimed to evaluate the antioxidant activity of different commercial antioxidant dietary supplements available in Portuguese market, by three *in vitr*o assays: scavenging activity against 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals, reducing power, and inhibition of lipid peroxidation using TBARS in brain homogenates. Moreover, some of the samples were mixed and further assayed in search of synergistic effects.

2. Materials and methods

2.1. Standards and reagents

2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). Standards trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and α-tocopherol were purchased from Sigma (St. Louis, MO, USA). Methanol and all other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Water was treated in a Mili-Q water purification system (TGI Pure Water Systems, USA).

2.2. Samples and samples preparation

Samples were dietary supplements commercially available and labeled with antioxidant potential. In order to confirm and compare their antioxidant activity, the samples were prepared using the formulation available: pill, capsule or bag (Table 1). Each formulation was weighted and dissolved in 200 mL of distilled water in order to obtain the concentration of the stock-solution. Pills and the inner part of the capsules were dissolved in distilled water, while bags were used to prepare infusions. Several dilutions of each sample were prepared to perform the antioxidant activity assays.

Some of the samples were mixed and further assayed in search of synergistic effects. Four mixtures were prepared: AAF + Res + EMCO (stock-solution 4.56 mg/mL) and the corresponding binary combinations: AAF + Res (stock-solution 3.60 mg/mL), AAF + EMCO (stock-solution 5.11 mg/mL) and Res + EMCO (stock-solution 4.99 mg/mL).

Table 1 Composition of the dietary supplements and concentrations of the stock-solution of each sample.

Sample	Composition	Formulation	Stock solution (mg/mL)
SACE	Disodium selenium (0.056%), vitamins A (retinol acetate: 0.74%), C (L-ascorbic acid: 22.5%) and E (α-tocopherol: 15%)	Pill (397 mg)	1.98
S200	Selenium: 200 µg, brewer's yeast	Pill (614 mg)	3.07
VB	Vitamins A, C (L-ascorbic acid) and E (D-α-tocopherol succinate), broccoli sprouts powder, red fruit (grape, blueberry, cranberry, cherry, strawberry and raspberry) combined extract, selenium (yeast).	Pill (578 mg)	2.89
BAPN	Vitamins A (retinol: 864 µg), B1 (thiamine: 1.8 mg), B2 (riboflavine: 2.8 mg), B3 (nicotinamide), B5 (pantothenic acid: 7.5 mg), B6 (pyridoxine), B7 (biotin: 100 µg), B11 (folacin: 200 µg), B12 (cyanocobalamin: 4.5 µg), C (ascorbic acid: 200 mg), D (calcipherol: 2.5 µg) and E (D-α-tocopherol: 30 mg), magnesium (75 mg), zinc (7.5 mg), Selenium (L-selenomethionine: 62.5 µg), chromium (yeast: 50 µg), manganese (2.5 mg), copper (1 mg)	Pill (1058 mg)	5.29
LLSC	Soy isoflavones, vitamin C, *Lycopersicon esculentum* extract, lactoproteins, soy lecitin, Lacto-licopene	Pill (737 mg)	3.68
KAG	Aged garlic extract, *Sylibum marianum* extract, green tea (powder), vitamins A (β-carotene), C (L-ascorbic acid) and E (α-tocopherol succinate), grape seed extract, pine bark extract, selenium (L-selenomethionine)	Capsule (431 mg)	2.16
SZCEA	Sodium selenite (0.02%), zinc sulphate (4.8%), vitamins A (β-carotene: 7.5%), C (calcium L-ascorbate: 12%) and E (D-α-tocopherol acetate: 12%)	Capsule (374 mg)	1.87
AAF	Vitamins A (β-carotene: 4.5 mg), C (calcium L-ascorbate: 500 mg) and E (D-α-tocopherol succinate: 134 mg and other tocopherols: 20 mg), L-cysteine chloridrate; food based antioxidants: powdered extracts of green tea (7.5 mg of polyphenols), red wine (4.5 mg of polyphenols) and Pycnogenol (3 mg of procyanidins), zinc glycinate (10 mg), taurine (50 mg), L-glutathione (50 mg), manganese glycinate (4 mg),powdered active plant base (Spirulina, *Ginkgo biloba*, *Sylibum marianum* and *Gotu kola* extracts), selenomethionine (50 µg), copper lysinate (1 mg) and riboflavin-5-phosphate (6 mg)	Capsule (744 mg)	3.72
Pyc	Pycnogenol (*Pinus maritima* bark extract): 30 mg	Capsule (247 mg)	1.24
Res	Resveratrol(*Polygonum cuspidatum* root extract): 200 mg	Capsule (695 mg)	3.48
GC	*Coffee arabica* seeds (whole cryogrinded powder): 1% caffeine	Capsule (288mg)	1.44
AA	Vitamin C (L-ascorbic acid) and E (α-tocopherol: 50%), green tea powder, rosemary leaf powder, grape extract, propolis alcoholic extract, *Pinus albicaulis*	Capsule (220 mg)	1.10
GBGT	Vitamin A (retinol acetate: 0.083%), C (L-ascorbic acid: 13.9%) and E (D-α-tocopherol: 5.6%), *Lycopersicum esculentum* fruit: 6.9%, *Gingko biloba* leaves (6.9%), *Camelia sinensis* (green tea): 1.9%; β-carotene: 0.7%.	Capsule (650 mg)	3.25
GM	Mangosteen 10:1 (*Garcinia mangostana*)	Capsule (848 mg)	4.24
VRFR	*Vitis vinifera* (red vine leaves: 35%), *Hibiscus sabdariffa* (flowers: 25%), *Pyrus malus* (fruit: 16%), orange and red fruits natural flavors	Bag (1500 mg)	7.50
EA	*Equisetum arvense* (30%), *Olea europaea* (30%), *Crataegus laevigata* (20%), *Mentha piperita* (20%)	Bag (1300 mg)	6.5
EMCO	*Equisetum arvense*, *Mentha spicata*, *Crataegus monogyna*, *Olea europaea*	Bag (1300 mg)	6.5

2.3. Antioxidant activity assays

2.3.1. General

The antioxidant activity of the individual and mixed samples was evaluated by DPPH radical-scavenging activity, reducing power and inhibition of lipid peroxidation using TBARS in brain homogenates. The sample concentrations providing 50% of antioxidant activity or 0.5 of absorbance (EC_{50}) were calculated from the graphs of antioxidant activity percentages (DPPH and TBARS assays) or absorbance at 690 nm (reducing power assay) against sample concentrations. The concentrations range was defined in order to allow percentages of antioxidant activity from ~10% to ~90% (sock-solution and successive dilutions). Trolox and α-tocopherol were used as standards.

2.3.2. DPPH radical-scavenging activity

This methodology was performed using an ELX800 Microplate Reader (Bio-Tek Instruments, Inc). The reaction mixture on 96 wells plate consisted of a solution by well of the different samples concentrations (30 µL) and methanolic solution (270 µL) containing DPPH radicals (6×10^{-5} mol/L). The mixture was left to stand for 30 min in the dark. The reduction of the DPPH radical was determined by measuring the absorption at 515 nm (Guimarães et al., 2010). The radical scavenging activity (RSA) was calculated as a percentage of DPPH discoloration using the equation: $\%RSA = [(A_{DPPH} - A_S)/A_{DPPH}] \times 100$, where A_S is the absorbance of the solution containing the sample, and A_{DPPH} is the absorbance of the DPPH solution.

2.3.3. Reducing power

The different concentrations of the samples solutions (0.5 mL) were mixed with sodium phosphate buffer (200 mmol/L, pH 6.6, 0.5 mL) and potassium ferricyanide (1% w/v, 0.5 mL). The mixture was incubated at 50 °C for 20 min, and trichloroacetic acid (10% w/v, 0.5 mL) was added. The mixture (0.8 mL) was poured in the 48 wells plate, as also deionised water (0.8 mL) and ferric chloride (0.1% w/v, 0.16 mL), and the absorbance was measured at 690 nm in the Microplate Reader described above (Guimarães et al., 2010).

2.3.4. Inhibition of lipid peroxidation using thiobarbituric acid reactive substances (TBARS)

Brains were obtained from pig (*Sus scrofa*), dissected, and homogenized with a Polytron in ice cold Tris–HCl buffer (20 mM, pH 7.4) to produce a 1:2 w/v brain tissue homogenate which was centrifuged at 3000g for10 min. An aliquot (0.1 mL) of the supernatant was incubated with the different concentrations of the samples solutions (0.2 mL) in the presence of $FeSO_4$ (10 mM; 0.1 mL) and ascorbic acid (0.1 mM; 0.1 mL) at 37 °C for 1 h. The reaction was stopped by the addition of trichloro acetic acid (28% w/v, 0.5 mL), followed by thiobarbituric acid (TBA, 2% w/v, 0.38 mL), and the mixture was then heated at 80 °C for 20 min. After centrifugation at 3000g for 10 min to remove the precipitated protein, the color intensity of the malondialdehyde (MDA)–TBA complex in the supernatant was measured by its absorbance at 532 nm (Ng et al., 2000). The inhibition ratio (%) was calculated using the following formula: Inhibition ratio (%) = [(A − B)/A] × 100%, where A and B were the absorbance of the control and the sample solution, respectively.

2.4. Statistical analysis

All the assays were carried out in triplicate in three different samples of each single supplement. The results are expressed as mean value ± standard deviation (SD). The statistical differences represented by letters were obtained through one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference post hoc test with $\alpha = 0.05$, coupled with Welch's statistic. The homoscedasticity of distribution was checked through Levene's test.

In addition, a linear discriminant analysis (LDA) was used as a supervised learning technique to classify the assayed antioxidant dietary supplements according to their antioxidant activity results. A stepwise technique, using the Wilks' λ method with the usual probabilities of F (3.84 to enter and 2.71 to remove), was applied for variable selection, verifying which canonical discriminant functions were significant. To avoid overoptimistic data modulation, a leaving-one-out cross-validation procedure was carried out to assess the model performance.

Moreover, the sensibility and specificity of the discriminant model were computed from the number of individuals correctly predicted as belonging to an assigned group (López et al., 2008 and Rencher, 1995). Sensibility was calculated by dividing the number

of samples of a specific group correctly classified by the total number of samples belonging to that specific group. Specificity was calculated by dividing the number of samples of a specific group classified as belonging to that group by the total number of samples of any group classified as belonging to that specific group. LDA statistical analysis and the other statistical tests were performed at a 5% significance level using the SPSS software, 18.0 (SPSS Inc.).

3. Results and discussion

The composition of the assayed dietary supplements is described in Table 1. Their selection was based in the different components included in the available formulations, either as single active components or in different combinations. The antioxidant components comprise lipophilic (e.g. vitamin E and β-carotene) and hydrophilic (e.g. vitamin C and polyphenols) molecules, natural extracts (e.g. Ginkgo biloba and Mentha spicata) or synthetic compounds (e.g. sodium selenite and zinc sulfate).

A wide range of methods have been used to screen the in vitro antioxidant capacity of foods and dietary supplements (Antolovich et al., 2002, Dávalos et al., 2003, Prior and Cao, 2000 and Moon and Shibamoto, 2009). Standard procedures regarding antioxidant capacity methods have been recommended (Dávalos et al., 2003, Frankel and Meyer, 2000, Frankel and Finley, 2008 and Prior and Cao, 1999), but this issue is still matter of debate.

Herein, three *in vitro* assays: scavenging activity against DPPH radicals, reducing power, and inhibition of lipid peroxidation using TBARS in brain homogenates were applied to evaluate the antioxidant activity of dietary supplements commercialized in Portugal.

Table 2 gives the results obtained for the antioxidant activity of individual and combined samples. As expected, results regarding antioxidant activity of the different commercial dietary antioxidant supplements show great variability, reflecting their diverse composition and concentrations

Table 2. Antioxidant activity of individual and combined samples of dietary supplements. In each column different letters mean significant differences ($p<0.05$).

Sample	DPPH scavenging activity EC_{50} (mg/mL)	Reducing power EC_{50} (mg/mL)	TBARS inhibition EC_{50} (mg/mL)
SACE	0.12±0.02 l	0.0337±0.0004 i	1.9±0.1 b
S200	2.9±0.1 c	2.96±0.05 b	2.8±0.1 a
VB	0.36±0.02 jk	0.0436±0.0005 i	0.047±0.002 j
BAPN	1.03±0.02 f	0.145±0.001 hi	0.071±0.005 j
LLSC	0.62±0.04 hi	0.352±0.004 f	1.55±0.04 e
KAG	0.76±0.05 gh	0.290±0.005 fgh	0.14±0.01 i
SZCEA	0.196±0.04 kl	0.42±0.04 ef	1.82±0.02 c
AAF	0.052±0.001 l	0.042±0.005 i	0.032±0.003 j
Pyc	0.46±0.05 ij	0.171±0.001 ghi	0.047±0.001 j
Res	1.6±0.1 e	0.68±0.01 d	0.051±0.001 j
GC	0.84±0.05 fg	0.355±0.003 f	0.9±0.1 g
AA	0.21±0.04 kl	0.0614±0.0005 i	0.049±0.004 j
GBGT	0.18±0.05 kl	0.556±0.002 de	1.70±0.04 d
GM	3.7±0.1 a	3.61±0.01 a	1.43±0.05 f
VRFR	3.2±0.4 b	0.98±0.02 c	0.46±0.01 h
EA	1.8±0.2 d	0.88±0.01 c	0.51±0.02 h
EMCO	0.73±0.02 gh	0.636±0.003 d	0.5±0.1 h
AAF+Res	0.069±0.001 l	0.0568±0.0002 i	0.043±0.003 j
AAF+EMCO	0.089±0.004 l	0.0690±0.0005 i	0.056±0.002 j
Res+EMCO	0.844±0.004 fg	0.31±0.01 fg	0.086±0.001 ij
AAF+Res+EMCO	0.14±0.02 l	0.0738±0.0003 i	0.07±0.01 j

EC_{50}: sample concentration providing 50% of antioxidant activity in DPPH and TBARS assays, or 0.5 of absorbance at 690 nm in reducing power assay.

Considering DPPH scavenging activity, AAF (0.052 ± 0.001 mg/mL) and SACE (0.12 ± 0.02 mg/mL) were the most powerful supplements; regarding TBARS inhibition, AAF (0.032 ± 0.003 mg/mL), VB (0.047 ± 0.002 mg/mL), Pyc (0.047 ± 0.001 mg/mL), AA (0.049 ± 0.004 mg/mL), Res (0.051 ± 0.001 mg/mL) and BAPN (0.071 ± 0.005 mg/mL) achieved the best results; in the case of reducing power, SACE (0.0337 ± 0.0004 mg/mL), AAF (0.042 ± 0.005 mg/mL), VB (0.0436 ± 0.0005 mg/mL) and AA (0.0614 ± 0.0005 mg/mL) revealed the highest antioxidant activity. In general, the obtained results confirmed the antioxidant potential of the assayed supplements, and some EC_{50} values are similar to those obtained with reference standards like α-tocopherol or trolox (Table 2).

The net effect of dietary antioxidants on health depends on intake levels, bioavailability, ability to scavenge ROS/RNS and synergistic effects (Liu, 2004 and Manach et al., 2005). It is well accepted that a cooperative mix of antioxidants in a balanced form appears to be more effective than high levels of one or a few antioxidants (Liu, 2004 and Wang et al., 2011). Therefore, four mixtures were assayed in search of synergistic effects: AAF + Res + EMCO and the corresponding binary combinations: AAF + Res, AAF + EMCO and Res + EMCO. Since some of the assayed supplements already contain several components, it was decided to include an infusion to avoid the unique use of pharmaceutical formulations. Besides, infusions are apparently better accepted by the consumers. EMCO was chosen due to its highest antioxidant activity among infusions. Furthermore, the sample that showed the highest antioxidant activity (AAF) was also included. Res was chosen in order to evaluate the influence of an antioxidant supplement which is composed mainly by a single molecule.

The types of interactions (synergistic, additive or antagonist) observed in the antioxidant activity of the dietary supplements mixtures are given in Table 3. For DPPH radical scavenging activity, as well as for reducing power assays, the mixtures were always synergistic (increase of antioxidant capacity). The synergistic effect predominated also in the TBARS inhibition assay, being observed in 75% of the mixtures; AAF + Res demonstrated an additive effect.

The results were also analyzed through LDA to evaluate if the observed differences were sufficient to differentiate the condensed dietary supplements (pills and capsules) from the bags formulations (infusions). All independent variables selected by the stepwise procedure of the discriminant analysis were statistically significant according to the Wilks'λ test ($p < 0.05$). The stepwise LDA was performed considering the results obtained in all the assayed antioxidant methods, ensuing in a discriminant model with two significant ($p < 0.001$ for the Wilks' λ test) discriminant functions. .

Table 3 Theoretical[a] *versus* experimental values of antioxidant activity of the combined samples of dietary supplements.

Mixtures	DPPH scavenging activity EC_{50} (mg/mL)			Reducing power EC_{50} (mg/mL)		
	Theoretical	Experimental	Effect	Theoretical	Experimental	E
AAF+Res+EMCO	0.78±0.04	0.14±0.02	S	0.18±0.03	0.07±0.01	
AAF+Res	0.81±0.05	0.069±0.001	S	0.041±0.001	0.043±0.003	

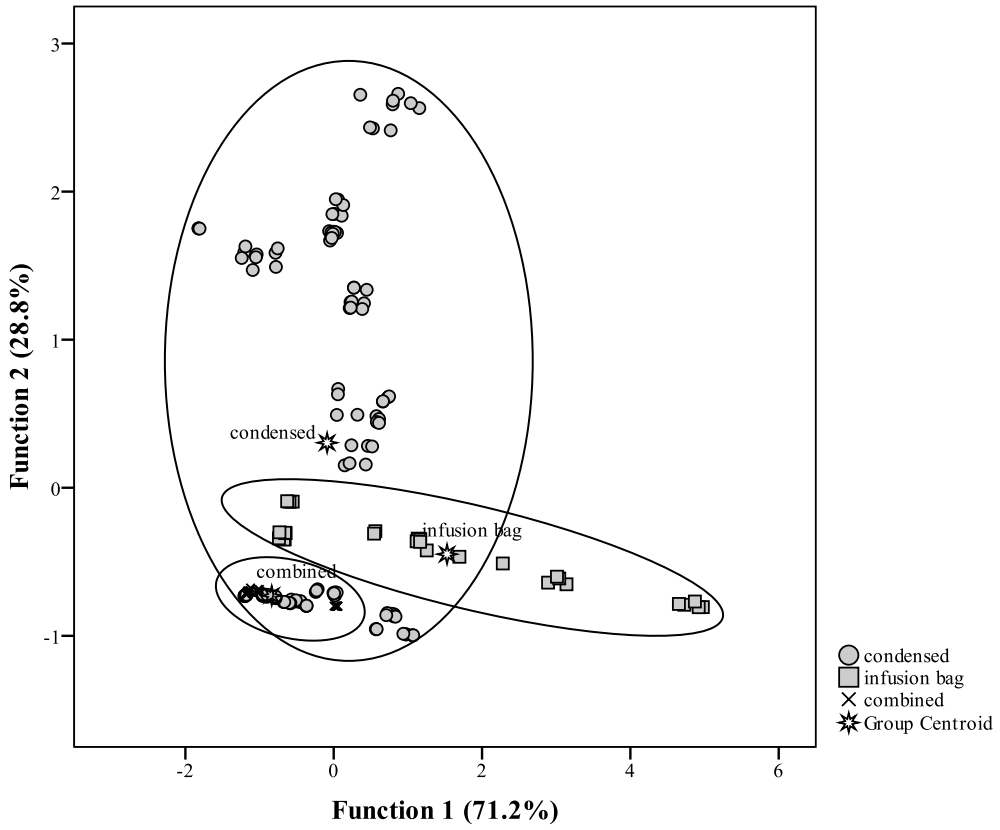

Figure 1 Canonical analysis of antioxidant dietary supplements (condensed- pills and capsules, infusion bags and combined- mixed samples) based on antioxidant activities (DPPH radical scavenging activity, reducing power, and TBARS formation inhibition).

These functions explained 100.0% of the variance of the experimental data (the first explained 71.2% and the second 28.8%. The first function (Fig. 1) revealed to be more powerfully correlated with DPPH scavenging activity, while TBARS inhibition was the most important variable for function 2. The model showed a satisfactory classification performance allowing to correctly classifying 70.8% of the samples for the original groups as well as for the cross-validation procedure. This separation is clearer in the case of infusion bags, demonstrating that the assayed combinations permitted to obtain mixtures with antioxidant potential more related with the condensed formulas (pills and capsules) than with the included infusion samples (bags).

Overall, AAF proved to have the highest antioxidant activity in all the assayed methods, providing the best results, either singly taken or included in mixtures. Furthermore, the mixtures containing this supplement revealed synergistic effects in 92% of the cases. The intake of antioxidant mixtures might provide some additional benefits, since the same antioxidant activity can be achieved with lower amounts of the chemical

compounds included in the pills or capsules. For the assayed combinations, the synergistic interaction was the main observed effect. Regarding LDA, the assayed dietary antioxidant supplements proved to have distinctive features, derived from being condensed (pills or capsules) or bags (infusions) formulas. Furthermore, it is relatively clear that the tested combinations retain an antioxidant profile highly similar to the presented by the condensed formulas included in those mixtures.

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Teas, dietary supplements and fruit juices: A comparative study regarding antioxidant activity and bioactive compounds

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Abstract

Nowadays, new emerging products claiming antioxidant properties are becoming more frequent. However, information about this topic in their labels is usually scarce. In this paper, we analyzed total phenolics, total flavonoids and ascorbic acid contents, as well as DPPH scavenging activity of several commercial samples, namely green tea and other herbal infusions, dietary supplements, and fruit juices, available in the Portuguese market. In general, beverages containing green tea and hibiscus showed higher phenolics contents (including flavonoids) and antioxidant activity than those without these ingredients. A borututu infusion presented the lowest concentrations of bioactive compounds and scavenging activity, due to the low recommended amount of plant to prepare the beverage. Some juices without antioxidant claims in the label presented similar values to those with it.

Keywords: Antioxidant activity; Ascorbic acid; Phenolic compounds; Tea; Dietary supplements; Juices

1. Introduction

Modern society is facing a global problem of chronic diseases, such as cardio and cerebrovascular ones, diabetes or cancer. Although human body has a natural defense system that protects itself against harmful effects of free radicals and reactive oxygen species, unhealthy eating habits and day-to-day stress may lead to a decrease in body defenses with deleterious health consequences (Lang & Heasman, 2005).

Phytochemicals (biologically active compounds from plants) have been receiving an increased attention due to their important and recognized health benefits, providing a potential protection against several injuries when moderately consumed (Oleszek, 2002). Indeed, antioxidant properties of several plant compounds (polyphenols, vitamin C, vitamin E, among others) have been widely publicized, since they may protect cellular systems of human body from oxidative damage through a variety of complementary and synergic mechanisms, and thus reduce the risk of chronic diseases (Kaur & Kapoor, 2001; Liu, Shi, Ibarra, Kakuda, & Xue, 2008; Serafini & Testa, 2009; Wojcik, Burzynska-Pedziwiatr, & Wozniak, 2010).

Modern consumers expect from food industry products easy and ready to consume, related with health and well-being. Besides, they also expect that processed foodstuffs have the same or more nutrients than natural foods.

Many recent studies have noted and highlighted the importance of antioxidant constituents of beverages, namely coffee (Alves et al., 2010), red wine (Martín, González-Burgos, Carretero, & Gómez- Serranillos, 2011), green tea (Lambert & Elias, 2010), fruit juices (Borges, Mullen, & Crozier, 2010), and flavored waters (Barroso, Noronha, Delerue-Matos, & Oliveira, 2011).

More and more, it is possible to find in the market several new products claiming their antioxidant properties. Indeed, these are becoming an important and emerging parameter to assess the quality of the product. Moreover, expansion of the global market and competition between food industries can make it part of the nutritional labeling. Nevertheless, and according to recent literature (Frankel & Finley, 2008) it has been highlighted that most phenolic compounds are poorly bioavailable and heavily metabolized in intestinal and hepatic cells and by the colonic microflora (with conjugation or elimination of phenolic -OH groups critical to the antioxidant activity). Indeed, the upper gastrointestinal tract might be the sole site where the antioxidant activity of phenolic compounds (in what concerns to direct scavenging of radical oxygen species) could be relevant. In this perspective, the term “antioxidant” should be used with precaution by food

manufacturers to correctly inform consumers. The knowledge of the antioxidant profiles of commercial food products is very important in order to compare them. In this work, we evaluated the antioxidant activity (against DPPH radical) of several commercial samples (fruit juices, green tea and other herbal infusions, as well as dietary supplements), all available in the Portuguese market. Within the compounds potentially involved, ascorbic acid, total phenolics, and total flavonoids were evaluated.

2. Material and methods

2.1. Reagents and equipments

Ascorbic acid, DPPH• (1,1-diphenyl-2-picrylhydrazyl) free radical, (-)-epicatechin, Folin-Ciocalteu's reagent, gallic acid, iodine, trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, a water-soluble derivative of vitamin E), and starch, were all purchased from Sigma-Aldrich (Steinheim, Germany). Ethanol of reagent grade, sodium acetate, sodium carbonate decahydrate, sodium nitrite, aluminum chloride, sodium hydroxide were purchased from Merck (Darmstadt, Germany). Spectrophotometric measurements were performed in a UV-visible spectrophotometer (UV-1800) from Shimadzu (Japan).

2.2. Samples

Samples (n=19) were randomly selected from local supermarkets and herbalist shops in the area of Oporto, Portugal. Essentially, products claiming antioxidant properties or containing antioxidant constituents were selected. A detailed description of samples is presented in Table 1.

2.3. Samples preparation

Samples as bags, leaves, roots or soluble granulates were used to prepare beverages according to the manufacturer's instructions, as described in Table 1. These and the remaining commercial beverages were all filtered, analyzed immediately for ascorbic acid content, and stored in the dark at -20ºC, for the remaining determinations. Diluted extracts of samples were prepared as necessary.

Table 1. Samples description and methods of beverages preparation.

Code	Samples Composition	Presentation	Method of beverage preparation
Teas and dietary supplements			
1	Green tea (*Camellia sinensis*) (100%)	Bags (2 g)	Infusion of a bag in 200 ml of hot water (75 ºC), during 5 min
2	Green tea (*Camellia sinensis*) (100%)	Dry leaves	Infusion of leaves (1.62 g) in 200 ml of hot water (75ºC), during 3 min
3*	Green tea (*Camellia sinensis*) extract (2%), sugar, dextrose, citric acid, ascorbic acid (0.225%)	Soluble granulated powder	Solubilization of 16.6 g of powder in 200 ml of hot water (75ºC)
4*	Green tea (*Camellia sinensis*) extract (100%)	200 ml syrup (liquid concentrated extract)	n.a.
5*	Red fruits juice powder (10%), green tea extract (6%), citric acid, fruit aromas, hibiscus extract (1.5%), grape seed (1%), ascorbic acid	Soluble powder	Solubilization of 5 g in 200 ml of hot water (75 ºC)
6	Green tea (*Camellia sinensis*) (70%), lemon herb (*Cymbopogon Citratus*) (20%), and algae (*Fucus Vesiculosus*) (10%)	Bags (1.5 g)	Infusion of a bag in 200 ml of boiling water (100 ºC), during 3 minutes
7	Green tea (83.3%), aromas (12%), hibiscus (2.4%), pineapple (2.3%)	Bags (2.3 g)	Infusion of a bag in 200 ml of hot water (75 ºC), during 5 minutes
8*	Borututu roots (*Cochlospermum angolensis*) (100%)	Dry roots	Infusion of roots (0.27 g) in 200 ml of hot water (75 ºC), during 3 minutes
9	Rooibos red tea leaves (*Aspalathus linearis*) (100%)	Bags (1.5 g)	Infusion of a bag in 200 ml of boiling water (100 ºC), during 4 minutes
Commercial fuits juices			
10	Pomegranate juice (16%), grape juice (4%), green tea extract (0,1%), carrot concentrated juice, hibiscus concentrated juice, ascorbic acid, citric acid	Tetra Pak package (1L)	n.a.
11	Blackberry juice (18%), grape juice (12%), red tea extract (0,11%), carrot concentrated juice, hibiscus concentrated juice, citric acid	Tetra Pak package (1L)	n.a.
12	Juice and pulp of raspberry (28%), juice and pulp of apple (22%), ascorbic acid	Tetra Pak package (330 ml)	n.a.
13	Red grape, raspberry, apple, strawberry, blackberry and cranberry juices, citric acid, ascorbic acid	Tetra Pak package (330 ml)	n.a.
14	Grape, cherry and blackberry juices, strawberry pulp, raspberry concentrated juice (40%), citric acid, ascorbic acid	Tetra Pak package (330 ml)	n.a.
15	Pineapple juice (35%) and grape juice (20%), citric acid, ascorbic acid	Tetra Pak package (330 ml)	n.a.
16	Juice and pulp of orange (23%), carrot (14%), mango (12%), lemon (1%)	Tetra Pak package (330 ml)	n.a.
17	Orange juice (27%), passion fruit (9%), lemon (2%), ascorbic acid	Tetra Pak package (330 ml)	n.a.
18	Orange juice (30%) and mango pulp (15%), ascorbic acid, citric acid	Tetra Pak package (330 ml)	n.a.
19	Tomato juice and pulp, citric acid, ascorbic acid	Glass bottle (200 ml)	n.a.

n.a., not applicable.
[a] Dietary supplement.

2.4. Ascorbic acid

A redox titration, involving an iodometric method, was used to perform ascorbic acid quantification. Briefly, 25.0 ml of sample were subjected to an iodimetric titration with 1 g/100 ml iodine solution, using a 1 g/100 ml starch solution as indicator. The endpoint was observed by the reaction of iodine with starch suspension, which produces a blue-black product. The standard ascorbic acid was used to plot the standard curve (linearity range = 0.2−1.0 mg/ml, r > 0.999). Ascorbic acid content was expressed as milligrams per 100 ml of beverage (or 10 ml of syrup).

2.5. Total phenolics

Total phenolics were spectrophotometrically determined according to the Folin-Ciocalteu procedure (Singleton & Rossi, 1965) with minor modifications (Alves et al., 2010). Briefly, 500 ml of a diluted sample was mixed with 2.5 ml of the Folin-Ciocalteu reagent (1:10) and 2 ml of a Na2CO3.10H2O solution (7.5 g/ 100 ml). The mixture was incubated at 45 ºC, during 15 min, and after 30 min at room temperature, absorbance readings at 765 nm were performed, against a reagent blank. A calibration curve for the standard gallic acid was used to obtain a correlation between sample absorbance and standard concentration (linearity range = 5−100 µg/ml, r > 0.999). Total phenolics concentration was expressed as milligrams of gallic acid (GA) per 100 ml of beverage (or 10 ml of syrup). The results obtained were afterward corrected in order to eliminate the ascorbic acid influence.

2.6. Total flavonoids

Total flavonoids content was determined by a colorimetric assay based on the formation of flavonoid-aluminum compound according to Barroso et al. (2011). Briefly, 1 ml of a diluted extract was mixed with 4 ml of ultrapure water and 300 µl of 5 g/100ml NaNO2 solution. After 5 min, 300 µl of 10 g/100ml AlCl3 solution were spiked, and after 1 min, 2 ml of 1mol/L NaOH and 2.4 ml of ultrapure water were also added. The solution was well mixed, and absorbance was read at 510 nm. Epicatechin was used to plot the standard curve (linearity range = 0−66 µg/ml, r > 0.999). Total flavonoids concentration was expressed as milligrams of epicatechin (E) per 100 ml of beverage (or 10 ml of syrup).

2.7. DPHH assay

The antioxidant activity of samples against the stable nitrogen radical DPPH• (2,2 diphenyl-1-picrylhydrazyl) was spectrophotometrically determined, according to Barroso et al. (2011), with minor modifications. Briefly, 200 µl of a diluted sample were mixed with 2.80 ml of an ethanolic solution of DPPH• (9.3 x 10^{-5} mol/L). The mixture was vigorously shaken and absorbance decrease was followed at 517 nm until a stable value was achieved. In this assay, the DPPH• is reduced to the corresponding hydrazine when reacts with hydrogen donors, such as an antioxidant, and lower absorbance values of the reactive mixture indicate higher free radical scavenging activity. A calibration curve was prepared with trolox (linearity range: 0 to19.6 µg/ml, r > 0.999) and results were expressed as milligrams of trolox per 100 ml of beverage (or 10 ml of concentrated extract).

2.8. Statistical treatment

Data are reported as mean standard deviation of three measurements. Statistical analyses were performed using the statistical package SPSS v 15.0 (SPSS for Windows; SPSS Inc., Chicago, IL). One-way ANOVA was used to compare two or more groups, and post-hoc Dunnett's test was performed for simultaneous paired comparisons. Differences at $p < 0.05$ (95% confidence level) were considered to be significant. Simple linear regression analysis was used to evaluate the relationship between compounds amount and DPPH scavenging activity.

3. Results and discussion

Several food products claiming antioxidant properties are commercially available. The samples selected for this study could be mainly separated in two large groups: teas/tea-based products and fruit juices.

Within the first group (Tables 1 and 2), samples of green tea (Camellia sinensis) for infusion (bags, sample 1; dry leaves, 2) and green tea dietary supplements (soluble granulated powder, 3; liquid concentrated extract or syrup, 4) were analyzed. Moreover, samples with a mixture of green tea and other antioxidant products, as red fruits/grape seed, lemon herb/algae, and pineapple/ hibiscus (5e7, respectively), as well as borututu roots (8) and rooibos red tea (9), were also considered.

Table 2. Bioactive compounds and antioxidant activity of teas and dietary supplements [a]

Code	Samples Composition	Ascorbic acid (mg/100 ml)	Total phenolics (mg GA/100 ml)	Total flavonoids (mg E/100 ml)	DPPH scavenging activity (mg T/100 ml)
1	Green tea (*Camellia sinensis*) (100%)	$42.8^{d} \pm 0.0$	$30.1^{d} \pm 0.5$	$8.4^{c} \pm 0.1$	$142.3^{a} \pm 33.8$
2	Green tea (*Camellia sinensis*) (100%)	$42.8^{d} \pm 0.0$	$23.9^{c} \pm 0.6$	$8.7^{d} \pm 0.0$	$61.6^{c} \pm 9.7$
3	Green tea (*Camellia sinensis*) extract (2%), sugar, dextrose, citric acid, ascorbic acid (0.225%)	$28.5^{b} \pm 0.0$	$46.8^{f} \pm 0.3$	$6.9^{b} \pm 0.0$	$92.9^{d} \pm 7.1$
4	Green tea (*Camellia sinensis*) extract (100%)	$30.7^{c} \pm 3.0$*	$14.1^{b} \pm 0.1$*	$8.8^{d} \pm 0.1$*	$25.7^{b} \pm 2.0$*
5	Red fruits juice powder (10%), green tea extract (6%), citric acid, fruit aromas, hibiscus extract (1.5%), grape seed (1%), ascorbic acid	$28.5^{b} \pm 0.0$	$46.8^{f} \pm 0.4$	$7.0^{b} \pm 0.0$	$136.5^{e} \pm 17.4$
6	Green tea (*Camellia sinensis*) (70%), lemon herb (*Cymbopogon Citratus*) (20%), and algae (*Fucus Vesiculosus*) (10%)	$53.5^{e} \pm 5.0$	$43.1^{e} \pm 0.5$	$10.6^{e} \pm 0.1$	$79.8^{cd} \pm 0.8$
7	Green tea (83.3%), aromas (12%), hibiscus (2.4%), pineapple (2.3%)	$85.5^{f} \pm 0.0$	$169.1^{g} \pm 1.8$	$29.1^{g} \pm 0.0$	$542.2^{f} \pm 39.6$
8	Borututu roots (*Cochlospermum angolensis*) (100%)	$14.3^{a} \pm 0.0$	$1.5^{a} \pm 0.0$	$0.34^{a} \pm 0.0$	$1.9^{a} \pm 0.3$
9	Rooibos red tea leaves (*Aspalathus linearis*) (100%)	$14.3^{a} \pm 0.0$	$22.0^{c} \pm 0.1$	$11.6^{f} \pm 0.0$	$24.0^{b} \pm 1.26$

Data followed by different letters within each column are significantly different at $p < 0.05$; *, results of sample 4 (syrup) are expressed in mg per spoon (10 ml); GA, gallic acid; E, epicatechin; T, trolox.

[a] Data are expressed as mean standard deviation (*n*=3).

In a general way, results revealed total phenolics as the main antioxidant compounds in these samples, followed by ascorbic acid, due to its natural presence in green tea (Cabrera, Artacho & Giménez, 2006) and in the other ingredients. However, in some samples, ascorbic acid might be intentionally added to the beverage by

manufacturers to protect the final product from oxidation, extending its durability. The higher content of ascorbic acid (43 mg/ 100 ml) was found in sample 7 (containing green tea, pineapple and hibiscus), while borututu and rooibos teas showed the lowest amounts (7 mg/100 ml, for both). Sample 7 was also the richest in total phenolics (167 mg GA/100 ml), including flavonoids (29 mg E/100 ml), and showed the strongest scavenging ability against DPPH radical (542 mg T/100 ml), while the borututu infusion (sample 8) presented the lowest values. Although borututu roots were described in the label as a phenolics-rich product, the recommended sample/volume ratio for the infusion preparation was very low (approximately 0.14 g/100 ml) compared to the other samples, what obviously influenced the obtained results. This product is normally used for its hepatoprotector properties (Aliyu, Okoye, & Shier, 1995), but in high doses it could lead to some toxicity.

The green tea syrup (sample 4), recommended to be consumed three times per day (unit dose of 10 ml), could be an interesting source of antioxidants, for those who do not appreciate plant infusions or tea-based beverages.

In what concerns to the second group of samples (Table 3), it is possible to observe that the juice containing green tea extract and hibiscus (sample 10), among other ingredients (pomegranate, grape and carrot), presented the highest content of total phenolics, including flavonoids, as well as the highest antioxidant activity. This goes in agreement with the results previously obtained for sample 7 (Table 2). In fact, green tea and hibiscus are described in literature as very good sources of phenolic compounds, comparatively with other medicinal plants (Rababah et al., 2011; Tsai, Tsai, Yu, & Ho, 2007). Green tea polyphenols include flavonols, flavandiols, flavonoids, phenolic acids, and essentially flavonols (commonly known as catechins). There are four kinds of catechins mainly find in green tea: epicatechin, epigallocatechin, epicatechin-3-gallate, and epigallocatechin gallate, being the latter the predominant one (Chacko, Thambi, Kuttan, & Nishigaki, 2010). Ramirez-Rodrigues, Plaza, Azeredo, Balaban, and Marshall (2011) identified hydroxybenzoic acids, caffeoylquinic acids, flavonols, and anthocyanins (delphinidin-3-sambubioside and cyanidin-3-sambubioside) in hibiscus extracts.

In a general way, total phenolics content found in the commercial fruit juices are in accordance with those reported in literature for similar products (Balasundram, Sundram, & Samman, 2006; Gardner, White, McPhail, & Duthie, 2000; Zulueta, Esteve, Frasquet, & Frígola, 2007). Samples 13 and 11 followed sample 10 in what concerns to antioxidant activity, probably due to the phenolic richness of red fruits. Additionally, sample 11 also contained hibiscus.

Table 3. Bioactive compounds and antioxidant activity of commercial fruit juices.[a]

Code	Samples Composition	Ascorbic acid (mg/100 ml)	Total phenolics (mg GA/100 ml)	Total flavonoids (mg E/100 ml)	DPPH scavenging activity (mg T/100 ml)
10	Pomegranate juice (16%), grape juice (4%), green tea extract (0,1%), carrot concentrated juice, hibiscus concentrated juice, ascorbic acid, citric acid	$43.8^{e} \pm 0.0$	$135.6^{i} \pm 0.0$	$18.1^{h} \pm 0.3$	$308.1^{i} \pm 14.3$
11	Blackberry juice (18%), grape juice (12%), red tea extract (0,11%), carrot concentrated juice, hibiscus concentrated juice, citric acid	$18.8^{a} \pm 0.0$	$68.3^{g} \pm 1.1$	$16.3^{g} \pm 0.4$	$149.4^{g} \pm 3.4$
12	Juice and pulp of raspberry (28%), juice and pulp of apple (22%), ascorbic acid	$25.0^{b} \pm 0.0$	$40.3^{c} \pm 0.4$	$6.2^{c} \pm 0.3$	$79.4^{d} \pm 4.9$
13	Red grape, raspberry, apple, strawberry, blackberry and cranberry juices, citric acid, ascorbic acid	$37.5^{d} \pm 0.0$	$74.6^{h} \pm 0.7$	$14.4^{f} \pm 0.0$	$173.1^{h} \pm 4.4$
14	Grape, cherry and blackberry juices, strawberry pulp, raspberry concentrated juice (40%), citric acid, ascorbic acid	$50.0^{f} \pm 0.0$	$49.3^{e} \pm 0.4$	$8.3^{d} \pm 0.0$	$61.9^{c} \pm 1.7$
15	Pineapple juice (35%) and grape juice (20%), citric acid, ascorbic acid	$37.5^{d} \pm 0.0$	$24.3^{a} \pm 0.4$	$5.5^{b} \pm 0.0$	$40.6^{b} \pm 2.6$
16	Juice and pulp of orange (23%), carrot (14%), mango (12%), lemon (1%)	$50.0^{f} \pm 0.0$	$44.1^{d} \pm 0.7$	$3.0^{a} \pm 0.0$	$98.1^{e} \pm 2.1$
17	Orange juice (27%), passion fruit (9%), lemon (2%), ascorbic acid	$31.3^{c} \pm 0.0$	$33.3^{b} \pm 1.1$	$11.7^{e} \pm 0.3$	$29.4^{a} \pm 4.4$
18	Orange juice (30%) and mango pulp (15%), ascorbic acid, citric acid	$37.5^{d} \pm 0.0$	$50.6^{e} \pm 0.7$	$5.2^{b} \pm 0.2$	$99.4^{e} \pm 3.5$
19	Tomato juice and pulp, citric acid, ascorbic acid	$50.0^{e,f} \pm 8.8$	$58.8^{f} \pm 1.1$	$6.5^{c} \pm 0.2$	$129.4^{f} \pm 5.9$

Data followed by different letters within each column are significantly different at $p < 0.05$; GA, gallic acid; E, epicatechin; T, trolox.

[a] Data are expressed as mean ± standard deviation (*n*=3).

The two samples containing both orange and mango (16 and 18) showed similar DPPH scavenging activities ($p < 0.05$), and near amounts of total phenolics. The lowest total phenolics content was observed in sample 15, composed by pineapple and grape juices, while the lowest total flavonoid amount was found in sample 16 (constituted by orange, carrot, mango, and lemon).

Ascorbic acid contents were generally variable (19-50 mg/ 100 ml), but different samples showed similar values. Once more, and especially in juices, ascorbic acid is used as food additive for preservation. Nevertheless, these values are in accordance with the reported values in literature for some commercial fruit juices (Gardner et al., 2000; Sánchez-Moreno, Plaza, Ancos, & Cano, 2003; Zulueta et al., 2007). Tomato juice (sample 19) showed intermediate antioxidant capacity and phenolic contents comparing with other samples, together with one of the highest ascorbic acid levels. Notice that other antioxidant compounds beyond those considered in this study could also influence the DPPH scavenging activity, as for example lycopene in the case of tomato (Liu et al., 2008).

4. Conclusions

In general, beverages containing green tea and hibiscus showed higher phenolic contents and antioxidant activities than those without these ingredients. Among all the samples (1-19), the borututu infusion presented the lowest concentrations of bioactive compounds and scavenging activity due to the low recommended amount to prepare the beverage.

Some juices without antioxidant claims in the label presented similar values to those with it. Also within juices, those containing blackberry and raspberry as main ingredients presented the lowest ascorbic acid levels. Results are in agreement with the low labelled information. This study shows the importance of these parameters to discriminate samples in what concerns to the antioxidant capability of the different beverages.

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Submitted

Total selenium content of food supplements by microwave digestion and HR-CS ETAAS. Label accuracy evaluation.

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Abstract

The determination of selenium in food supplements is of major interest, due to the low range between beneficial and toxic effects of this element. The total selenium contents of eight different commercially available food supplements were determined. Microwave-assisted acid digestion was used for sample solubilisation and selenium was determined by high-resolution continuum source atomic absorption spectrometric with electrothermal atomization (HR-CS ETAAS). The electrothermal behaviour of selenium in the presence of different chemical modifiers, and pyrolysis and atomization temperatures, were optimized. Palladium nitrate-magnesium nitrate was selected as the matrix modifier. The optimum pyrolysis and atomization temperatures were 1050 °C and 2000 °C, respectively. The LOD and LOQ for selenium were 0.10 and 0.34 µg g^{-1}, respectively. The intra-day precision showed a 3.2 % RSD and inter-day precision RSD did not exceeded 6.7 %. The accuracy of the method was checked with a certified reference material and good agreement was found between measured and certified total selenium content ($p > 0.05$). The total selenium contents were determined and compared with the labelled values. Selenium levels food supplements ranged from 15.4 ± 0.9 to 205.3 ± 9.9 µg/ unit. These results were in good agreement with the average levels claimed on product labels, with an error lower than ±15%. Also, all supplements were in compliance with the recent recommendations made by the European Community regarding the acceptable difference

between labelled and measured values for minerals and vitamins in food supplements, fixed in -20% to +45% of the declared on label.

***Keywords*:** Food supplements; Selenium; High-resolution continuum source electrothermal atomic absorption spectrometry; Label accuracy.

1. Introduction

In the European Union (EU), the Food Supplements Directive [1] defines 'food supplements' as concentrated sources of nutrients or other substances with a nutritional or/and physiological effect whose purpose is to supplement the normal diet. They are marketed in dose form (capsules, tablets, pills, powders, liquids, etc.), alone or in combination, and are designed to be taken in measured small unit amounts [1].

Food supplements are generally used to overcome nutritional deficiencies, prevent or reduce the risk of disease, and/or to promote general well-being. Generally, consumers assume these products as natural and safe, using them in addition to, or as a replacement for, or alternative to pharmaceuticals. However, food supplements, unlike pharmaceutical drugs, do not require approval for safety and efficacy prior to their marketing. Manufacturers and/or distributors need only to notify the competent authority before marketing their product, and are responsible to ensure its compliance with the requirements of applicable legislation both in terms of safety and of consumer information [1]. With the widespread use of food supplements, it is essential to ensure the safety of these products for human consumption. There have been reports of the presence of impurities and the adulteration of several food supplements, lack of batch-to-batch consistency, and misformulated products [2, 3, 4].

Selenium is an essential trace element required for the normal growth, development and metabolism of both man and animals [5]. Selenium constitutes an integral part of important selenoproteins, including glutathione peroxidase (GSHPx), an antioxidant enzyme that protects cell membranes from free radicals damage, iodothyronine deiodinases, involved in the thyroid hormone metabolism, and thioredoxin reductase that, in conjunction with the compound thioredoxin, participates in the regeneration of several antioxidants from their oxidized forms, regulating cell growth and viability [6, 7].

Currently, the Recommended Dietary Allowance (RDA) for selenium is 55 µg/ day for healthy adult men and women, the estimated requirement necessary for maximization

of plasma GSHPx activity [8]. However, clinical evidence reports that intakes of selenium in excess of the RDA (200–300 µg/ day) may prevent certain cancers [9, 10] and cardiovascular disease [11], and improve immune response and male fertility [12, 13]. In the United States, the Food and Nutrition Board of the Institute of Medicine [8] as derived a Tolerable Upper Intake Level (UL) of 400 µg selenium/ day for adults while the European Community (EC) Scientific Committee on Food (SCF) has set a UL of 300 µg/ day for the European Union (EU) [14]. The selenium content of foods and fodders depends of their geographical origin and the respective soil selenium content and availability. Consequently, the selenium intake by humans varies considerably between countries and regions [15]. Some European countries, including Portugal, register selenium dietary levels below RDA guidelines [16, 17, 15]. Although overt selenium deficiencies are rare, suboptimal selenium status can lead to cancer and heart disease, and an impaired immune system [18]. Supplementation, therefore, is becoming a common practice among consumers of developed countries to compensate for dietary deficiencies and/or to prevent certain cancers and aging. However, in view of the narrow range between deficiency, essentiality and toxicity of selenium in human nutrition, and the documented cases of intoxication caused by selenium supplements, makes particularly important the control of these products.

Selenium has been determined in food supplements using different analytical techniques including inductively coupled plasma mass spectrometry (ICP-MS) [19], cathodic stripping voltammetry (CSV) [20], hydride generation atomic fluorescence spectrometry (HG-AFS) [21, 22], and electrothermal atomic absorption spectrometry (ETAAS) [23,24]. Line source- ETAAS has been extensively and successfully employed for the elemental analysis of several matrices [25] due to its versatility, low limits of detection, and selectivity. Recently, High-Resolution Continuum Source Atomic Absorption Spectrometry (HR-CS AAS) has extended the capabilities of conventional AAS methods. Novel features include a high-intensity xenon short-arc lamp used as a continuum radiation source and a high resolution double echelle monochromator, which allows performing fast-sequential multi-element measurements, and a linear charge-coupled device (CCD) array detector. The CCD contains 588 pixels, 200 of which are used for the monitoring the analytical signal, but also allows the simultaneous visualization at high resolution of the spectral environment around the analytical line, and in the background correction. Spectral interferences can be detected promptly and corrected if necessary, reducing noise levels and improving the detection limits [26]. HR-CS AAS has been employed for elemental analyses of diverse matrices [27, 28, 29]. Recently,

Krawczyk [30] determined macro and trace elements in multivitamin dietary supplements by HR-CS AAS, with slurry sampling.

The aim of this work was to optimise and validate a method using microwave-assisted acid digestion and HR-CS ETAAS to quantify total selenium contents in commercially available food supplements and to compare the results against the amounts referred on the supplement label.

2. Materials and Methods

2.1 Equipment and HR-CS ETAAS measurement conditions

Selenium determination was performed using an Analytik Jena contrAA 700 (Analytik Jena, Jena, Germany) high-resolution continuum source atomic absorption spectrometer. The equipment has a transversely heated graphite furnace, a high-intensity xenon short-arc lamp (XBO 301, GLE, Berlin, Germany), a high-resolution double monochromator, and a charge-coupled device (CCD) array detector and an MPE 60 autosampler. Pyrolytically coated graphite tubes with integrated platform (Analytik Jena, Jena, Germany) were used exclusively. Argon of 99.95% purity (Linde Sogás, Portugal) was used as purge gas. The optimised electrothermal program used for selenium determination in samples and the reference material is shown in Table 2. Quantification was performed using 5 µL matrix modifier volume and 10 µL sample volume, sequentially pipetted by the auto sampler. The measurements were performed in a spectral interval of 0.2209 nm (200 pixels) around the primary selenium line 196.0267 nm (pixel 101). The integrated absorbance (A_{int}) was optimised and it was selected the values obtained for seven pixels (the central pixel ± 3), corresponding to the wavelength range of 7.7 pm. A dynamic background correction, based on those pixels that do not significantly differ from baseline noise, was used. Analytical blanks and standards were checked routinely to check instrument performance. Four replicate measurements of absorbance were carried out for all solutions.

Samples digestion was performed with a MARS X 1500W Microwave Accelerated Reaction System (CEM Corp., Mathews, NC, USA) and 100 mL Teflon HP-500 Plus closed-system vessels (CEM Corporation, Matthews, NC).

All glassware and plastic materials were washed with an appropriate detergent, immersed in 10% HNO_3 for 24 h and rinsed with ultrapure water, prior to use.

2.2 Reagents and solutions

Ultrapure water from a Simplicity 185 system (resistivity 18.2 MΩ.cm; Millipore, Belford, USA) was used for the preparation of samples and standards. Chemicals were of analytical reagent grade unless otherwise stated. Suprapur® grade nitric acid (65%) and hydrogen peroxide (30%) were obtained from Merck (Darmstadt, Germany). Selenium working standards were prepared by dilution of a 1000 mg L^{-1} selenium stock solution (Panreac, Barcelona, Spain). The Pd and Mg modifier solutions were made by dilution with water from commercially available stock solutions, 10.0 ± 0.2 g L^{-1} Pd in 15% (v/v) HNO3 (Merck, Darmstadt, Germany) and 10.0 ± 0.2 g L^{-1} Mg in 15% (v/v) HNO_3 (Panreac, Barcelona, Spain), respectively. A 1% (m/v) nickel nitrate solution, used as chemical modifier, was prepared by dissolving an appropriated amount of $Ni(NO_3)_2 \cdot 6H_2O$ (Merck, Darmstadt, Germany) in water.

Table 1. Description of the analysed food supplements.

Sample	Description	Claims	RDD
A	**Selenium (L-selenomethionine)** (yeast free).	No claim	1-2 tablets
B	**Selenium (L-selenomethionine).** Aged garlic extract, *Sylibum marianum* extract, green tea (powder), vitamins A, C and E, grape seed extract, pine bark extract.	Antioxidant and anti-aging	4 capsules
C	**Selenomethionine.** Vitamins A, C, and E, L-cysteine chloridrate, powdered extracts of green tea, red wine and pycnogenol, zinc glycinate, taurine, L-glutathione, manganese glycinate, powdered active plant base (Spirulina, *Ginkgo biloba*, *Sylibum marianum* and *Gotu kola* extracts), copper lysinate, riboflavin-5-phosphate.	Antioxidant	2 tablets
D	**Selenium (L-selenomethionine)**. Vitamins A, B1, B2, B3, B, B6, B7, B11, B12, C, D and E, magnesium, zinc, chromium, manganese, copper.	Antioxidant	1 tablet
E	**Selenium: brewer´s yeast.**	Helps support the immune system	1 tablet
F	**Selenium (yeast)**.Vitamins A, C and E, broccoli sprouts powder, red fruit (grape, blueberry, cranberry, cherry, strawberry and raspberry).	Antioxidant	1 tablet
G	**Disodium selenium**. Vitamins A, C and E.	No claim	1 tablet
H	**Sodium selenite**. Zinc sulphate, vitamins A, C and E.	Antioxidant	1 capsule

2.3 Sampling and sample preparation

Eight different food supplements containing selenium, for adult consumption, were purchased from local retail and herbal stores. The samples were selected to encompass

different selenium species (organic and inorganic) and formulations (tablet or capsule dosage). The supplements were designated as A, B, C, D, E, F, G, and H, respectively. The specifications of the selected supplements, according to the manufacturer, are summarised in Table 1.

Ten tablets or capsules were taken from each product, and then crushed and homogenised manually in a mortar, after carefully removing tablets film coats, if present, and the hard-gelatine in capsules. Powdered samples were stored in screw-capped vials and kept at 4°C until analysis.

Table 2. Optimized furnace program used for the determination of selenium by HR-CS ETAAS.

Step	Temperature (^{o}C)	Ramp (^{o}C/s)	Hold time (s)	Ar flow rate L/min^{-1}
Drying	90	3	20	Max
Drying	110	5	10	Max
Pyrolysis	1050	300	10	Max
Atomization	2000	1500	4	Stop
Cleaning	2450	500	4	Max

2.4 Microwave-assisted digestion

Approximately 0.2 g of each powdered sample was weighed into 100 mL microwave Teflon vessels and 9 mL of concentrated nitric acid and 1 mL of hydroxide peroxide were added to each vessel. The vessels were left open for 15 minutes before sealing to allow samples to predigest, and were then positioned inside the microwave digestion system for a three-step microwave temperature program. First, samples were digested at 50 °C, with 3 min ramp to temperature, for 3 minutes. Subsequently, samples were irradiated to a temperature of 90 °C, with 10 min. ramp and 10 min hold. Finally, samples were digested at 190°C for 20 minutes, using a ramp time of 10 minutes. Once the vessels were cooled, the digested samples were transferred to volumetric flasks and diluted to 15 mL with Milli-Q water. One reagent blank was run with each batch of samples. All the experiments were performed in triplicate.

A certified reference material SELM-1 (selenium-enriched yeast) used in this work to validate results, was obtained from the National Research Council of Canada (NRCC) (Ottawa, Québec, Canada) and was submitted to the same procedure applied to samples.

2.5 Statistical analysis

Data are reported as mean ± standard deviation. One-sample t-test was used to compare means. Statistical analysis was carried out using IBM SPSS Statistics for Windows, version 21.0 (IBM Corp., Armonk, New York). Significant differences were considered when $p < 0.05$.

3. Results and discussion

3.1 Selenium measurements by HR-CS ETAAS

The electrothermal behaviour of selenium in the presence of the chemical modifiers, and pyrolysis and atomization temperatures, were optimized to maximize absorbance signals and to minimize backgrounds and matrices interferences.

The determination of selenium by ETAAS requires the use of adequate chemical modifiers to avoid elemental volatilization during the different stages of the electrothermal process, and the consequent loss of analyte. Nickel (1%), palladium (0.1%), magnesium (0.1%), and palladium-magnesium (0.1% Pd+0.05% Mg) nitrates solutions, prepared in water from the respective modifier stock solutions, were tested as chemical modifiers for the determination of selenium by HR-CS ETAAS. A 50 µg L^{-1} selenium standard solution and digested samples were analyzed with the modifiers, using the default cookbook values. The best sensitivity was achieved with 5 µL of the palladium nitrate-magnesium nitrate (0.1% Pd+0.05% Mg) mixed modifier, and was further used for the optimization of the electrothermal program.

The optimization of pyrolysis and atomization temperatures was carried out automatically, using a 50 µg L^{-1} selenium standard solution and four digested sample solutions, in order to assure equal responses from selenium originated from selenite, selenomethionine and selenized yeast, present in the supplements. The optimum pyrolysis and atomization temperatures were 1050 °C and 2000 °C, respectively, and were used for all analyses. The optimized electrothermal program used for selenium determination by HR-CS ETAAS is shown in Table 2.

The spectral environment, only available with this recent instrument, allowed detecting two selenium peaks (196.027 nm and 196.026 nm). Since the sensitivity and the precision of the methods depends on the wavelength integrated absorbance, and selenium has two overlapping peaks, it was necessary to evaluate the integrated

absorbance of a standard solution for different number of pixels. The best precision was achieved for 7 pixels, i.e. from central pixel (196.027 nm) ± 3.

3.2 Method validation

Under the optimized conditions, the linear range, the limits of detection (LOD) and quantification (LOQ), the precision and the accuracy for the determination of selenium by HR-CS ETAAS were assessed. Calibration curves were obtained by using five selenium standard solutions (0, 12.5, 25, 50, 75, and 150 µg L^{-1}). Correlation coefficients better than 0.998 were obtained. The LOD and LOQ were calculated as 3 and 10 times the standard deviation estimated from the regression line divided by the slope of the calibration curve, respectively, and taking into account the dilutions done, and are expressed in µg g^{-1}. The LOD and LOQ for selenium were 0.10 and 0.34 µg g^{-1}, respectively. The intra- and inter-day precisions were evaluated by analysing one sample (supplement E) six times, under the same experimental conditions and on the same day, and on three different days. The intra-day RSD was 3.2 % and the inter-day RSD did not exceeded 6.7 %.

The accuracy of the method was verified by the analysis of the certified reference material SELM-1, Selenium Enriched Yeast, which has a total certified amount of selenium of 2059 ± 64 µg g^{-1}. The material was analysed in triplicate by the proposed method and an average concentration of 1999 ± 41 µg g^{-1} of total selenium was obtained (98.6 ± 2.8 % recovery). No significant differences were found between the certified and the experimental values ($p > 0.05$), confirming the accuracy of the method for the determination of total selenium food supplements with added selenium.

3.3 Quantification of total selenium in Food Supplements

The market offers a plethora of food supplements with added selenium. In this work, samples were selected to encompass supplements with different selenium chemical species and different formulations. Samples A, B, C, and D contained L-selenomethionine, selenized yeast was present in samples E and F, and samples G and H contained sodium selenite. The selected samples comprised single-component selenium supplements and multi-ingredient formulations that, besides selenium, could contain minerals, vitamins, amino acids, and other antioxidants, in capsules or tablets (Table 1). The concentrations of total selenium were determined by HR-CS ETAAS, after microwave-assisted digestion of samples. Results are summarized in Table 3. Selenium levels in samples ranged from 15.4 ± 0.9 to 205.3 ± 9.9 µg/ unit. When compared with the

declared value, the total selenium contents obtained were in good agreement with the label, with an error lower than ±15%.

Table 3. Total selenium contents in 8 food supplements. Experimental results as mean value ± standard deviation (n=3).

Sample	Declared Se content/unit (µg)	Measured Se content/ unit (µg)	% difference from labelled Se/unit level*
A	100	87.9±8.0	-12
B	17.5	15.4±0.9	-10
C	25	21.6±1.7	-14
D	62.5	70.6±3.3	+13
E	200	205.3±9.9	+3
F	100	102.0±7.3	+2
G	100	100.6±6.3	+1
H	50	49.4±4.8	-1

*Percent difference of label claim calculated as (measured Se content/ unit - Declared Se content/unit)/ Declared Se content/unit*1000

In 2012, the European Community Commission Standing Committee on the Food Chain and Animal Health has published guidance which established a tolerance threshold for nutrient values declared on nutrition labelling, defining this "tolerance threshold" as the acceptable differences between declared nutrient values on the label and those determined during official controls. The mineral content declared on label in food supplements should be in the range of -20 % to +45 % of the value declared [31]. According to this guidance, the differences between claimed and determined total selenium in all supplements are acceptable.

The results obtained in our study are comparable to the difference range (–19% to +23%) reported by Feifer et al. [32] after analysing five selenium supplement brands used for prostate disease. In a previous study, eight different commercial supplements were analysed by GFAAS and results were found to be in good agreement with label contents [23]. B'Hymer et al. [19] analyzed six different brands of yeast-based supplements for total selenium contents using microwave digestion and ICP-MS. Reported results ranged from 91 to 111% of the label claim. A few studies have identified discrepancies between stated and actual contents in selenium supplements. Valiente et al. [33] analysed 3 different selenium supplements brands for total selenium by ETAAS and found significant differences between tablets and between batches of the same brand, with differences over 300% in one brand. More recently, a work by Stibilj et al. [34] revealed that 2 of the 9

food supplements containing selenium analysed by HG-AFS did not comply with U.S. Pharmacopoeia and that there was variability between different batches of the same brand. In the same year, Veatch et al. [35] analysed 15 food supplements based on selenized yeast and selenate by neutron activation analysis technique and verified that there were significant differences between the labelled and determined selenium levels.

The RDD from producers was correlated with the RDA and the UL guidelines for selenium. When consumed according to the RDD, all supplements exceed the RDA (55 µg/ day), with exception of supplement C. Nevertheless, this would not appear to present a realistic hazard of a consumer exceeding the UL (300-400 µg/day) [14, 15], considering the low average selenium daily intake by the Portuguese population (inferior to the RDA) [17].

In recent decades the chemical species and levels of selenium present in food supplements has been the target of several studies. The control of selenium levels in these products is important not only to control label accuracy to avoid frauds, but also because selenium is toxic in very small amounts. In the last years, a few cases of intoxication caused by selenium nutritional supplements have been reported. Although improperly formulation is uncommon, the consequences can be serious. In 1996, Clark et al. [36] reported a case of intoxication in a man taking a nutritional supplement for fatigue. Although the product label specified 5 µg of Se per six tablets, subsequent analysis revealed a level 500-1000 superior to the declared amount per tablet. In May 2008, 201 cases of selenium poisoning were reported by the FDA in the USA. Poisoning was caused by the ingestion of a misformulated liquid food supplement containing almost 200 times the intended concentration of selenium [3].

3.4 Conclusions

Considering the widespread use of food supplements containing selenium, and the fact that these products are not required to be compliant with the same standards enforced for pharmaceuticals, it is essential their monitoring to ensure the safety of these products for human consumption. Microwave-assisted digestion followed by analysis by HR-CS ETAAS was found to be a suitable procedure for the determination of total selenium in food supplements. The analytical method was proven to be accurate and precise, and the determined detection and quantification limits are adequate for the routine analysis of selenium in food supplements, since selenium is usually present in food supplements at concentrations >10 µg g^{-1}. Generally, the total contents of selenium were in good agreement with the labelled average levels, with an error lower than ±15%.

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2.2– Suplementos Alimentares com Ácidos Gordos Ómega-3

D. Label compliance in omega-3 dietary supplements: oil, fatty acids and vitamin E contents analysis*. submitted*

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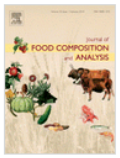

Label compliance in omega-3 dietary supplements: oil, fatty acids and vitamin E contents analysis

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Abstract

This study aimed to verify the conformity of the label information of 16 omega-3 food supplements, regarding their oil, fatty acids and vitamin E contents. Fish oil, krill or vegetable oils are present as components on the analysed products, in a single or blend form. The levels of oil, EPA, DHA, ALA and vitamin E were determined and compared with label information. Significant differences between the experimental data and the displayed information on the label were verified for the majority of the studied samples. These results stress the need to a more effective labelling control of this type of products, in order to better inform the consumers.

Keywords: Omega-3 fatty acids; ALA; DHA; EPA; Vitamin E; Food supplements.

Abbreviations: n-3 PUFA, omega-3 polyunsaturated fatty acids; ALA, α-linolenic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FAME, fatty acid methyl esters.

1. Introduction

The omega-3 fatty acids (*n*-3 FA) are a family of polyunsaturated fatty acids (PUFA) commonly found in marine and a few vegetable oils. The most important *n*-3 PUFA involved in human nutrition are α-linolenic acid (ALA, 18: 3 *n*-3), and the long-chain *n*-3 polyunsaturated fatty acids (LC *n*-3 PUFA) eicosapentaenoic acid (EPA, 20: 5 *n*-3), and docosahexaenoic acid (DHA, 22: 6 *n*-3) (Almeida et al., 2014).

Epidemiological and clinical studies have shown that regular consumption of oily fish and omega-3 fatty acids decreases the risk of cardiovascular disease (CVD), including fatal coronary heart disease (CHD) and sudden cardiac death (Mozaffarian & Wu, 2011). Based on this evidence, most international groups and organizations recommend to the general population a daily intake of at least 250 mg of LC *n*-3 PUFA (mostly EPA and DHA), preferably through consumption of oily fish (1–2 servings per week) (EFSA, 2010; FAO/WHO, 2008; Lichtenstein et al., 2006). Despite consumer awareness of the health benefits of *n*-3 PUFA, in most Western countries the estimated intake levels are lower than the currently recommended (Abreu et al., 2012; Lucas et al., 2009). Barrier factors for consuming fish and/or seafood have been identified and include, among others, taste preferences, price, environmental concerns, ethical reasons, and difficulties in preparation (Brunso et al., 2009; Trondsen et al., 2003). In those cases, supplementation may offer an effective way to increase *n*-3 PUFA intake and status.

Several products containing *n*-3 PUFAs are available in the market, and typically consist of fish, krill, algae or flaxseed oils or oil blends. *n*-3 PUFA in food supplements are delivered in various forms including soft gel capsules, oils and emulsions, and are available from multiple channels: retail stores, health stores, herbalists, in the internet, and pharmacies. Unlike medicines, which must be proven both safe and effective through clinical trials, dietary supplement manufacturers are responsible for the safety of their products (EC, 2013). Thus, sometimes these products lack consistency in dose and quality, according to several reports (Kolanowski, 2010; Opperman et al., 2011; Tatarczyk et al., 2007).

Foods containing lipids are susceptible to oxidation during processing and storage. *n*-3 PUFAs are naturally highly unstable and prone to oxidation, due to their unsaturated nature. The oxidation of oils give rise to volatile compounds that confer undesirable taste and smell, loss of nutrients, and the development of toxic lipid oxidation products that might be harmful for humans (Kanner, 2007). These compounds are associated with the rancidity which contributes to consumer rejection of the products. Common complains to fish oils are the presence of unpleasant fishy taste and smell, as well as digestive issues,

namely fishy eructation. Vitamin E is considered to be a major lipophilic antioxidant (Bramley et al., 20009) and it is commonly added to *n*-3 PUFA supplements to prevent oxidation and extend shelf life. The main function of vitamin E is to act as a primary antioxidant that scavenges lipid peroxyl radicals and neutralizes reactive oxygen and nitrogen species (Nelis et al., 2000). In humans, vitamin E has been associated with the prevention or delay of chronic diseases caused by oxidative stress including cardiovascular disease, cancer, cataracts, and Parkinson's disease (Bramley et al., 2000; Nelis et al., 2000).

The human body is not able to synthesise vitamin E and must obtain it through diet or supplementation. The Recommended Dietary Allowances (RDA) for vitamin E (α-tocopherol) for men or women older than 14 years is 15 milligrams (or 22.4 IU) (IMFNB, 2000).

Currently, to our knowledge, there is no available data on the evaluation of omega-3 supplements (from different origins) commercially available on the Portuguese market, regarding the quality and safety of these products. Thus, the aim of this study was to analyse 16 omega-3 supplements, concerning their FA profile, in particular, the EPA, DHA and ALA contents and also to quantify vitamin E amounts. Moreover, a comparison regarding these constituents was carried out, relating the analysed values with the manufacturers label information, important for the quality control and safety of these products.

2. Materials and methods

2.1. Reagents and standards

FAME 37 standard mixture was purchased from Supelco (FAME 37, Bellefonte, PA, USA). Tocopherols (α, β, γ, and δ) and tocotrienols (α, β, γ, and δ) were from Calbiochem (La Jolla, CA). The internal standard tocol was obtained from Matreya Inc. (Pennsylvania, USA). HPLC grade *n*-hexane was obtained from Merck (Darmstad, Germany) and 1,4-dioxane from Fluka. Butylated hydroxytoluene (BHT) was used as antioxidant and was from Aldrich (Madrid, Spain). All other chemicals were from analytical grade.

2.2. Samples

Sixteen oil supplements were obtained from health and retail stores in Porto, Portugal, and analysed for its fatty acids composition and vitamin E contents. Twelve samples are fish oils, were designated as FO and numbered from 1 to 12; one sample contained krill oil (KO), and the remaining three brands were blends of fish oil and vegetable oils (BL1, BL2, and BL3). All products were encapsulated in soft gel capsules. Detailed information about the analysed food supplements trade name, origin, ingredients, and the recommended daily dose (RDD) is presented in Table 1.

For sample preparation, six capsules of each brand were weighed, opened and the oil squeezed into a clean vial. The empty capsule was then washed with hexane, wiped and weighed again. Aliquots from the pooled oil were transferred to screw-capped vials for further analysis.

Table 1. Trade name, country of origin, oil source, and manufacturer recommended daily dose (RDD) of analysed dietary supplements.

Sample	Name	Oil source	Recommended daily dose (RDD)
FO1	ERGY 3 (France)	Sardines and anchovies oil	4
FO2	MorEPA (Belgium)	Deep sea fish oil	1
FO3	Omega 3 áreaviva (Portugal)	Polyunsaturated omega-3 fatty acids	4
FO4	Omega 3 Family (Belgium)	Fish oils	4
FO5	Aquamarine (USA)	Fish oil and cod liver oil	2
FO6	Omega 3 Mood (USA)	Fish oil concentrate (anchovy, sardine, mackerel, herring, salmon, tuna)	2
FO7	Omega 3 KAL (USA)	Fish oil from cold water fish	2
FO8	Omega 3 Concentration (Portugal)	Fish oil	1
FO9	Fish Oil Concentrate (USA)	Concentrated fish oil	1
FO10	Salmon Oil (Portugal)	Salmon oil	3
FO11	Omega 3- Fish Oil (Portugal)	Salmon oil	2
FO12	Omega-3 Double Strengh (USA)	Fish oil concentrate	4
KO	Physalis Krill Omega 3 (Belgium)	Krill oil	2
BL1	All-in-Fit Omega 3+Omega 6+Omega 9 (Portugal)	Fish oil; flaxseed oil; sunflower seed oil	3
BL2	Omega 3-6-9 Forma+ (Portugal)	Evening primrose oil; salmon oil; flaxseed oil	2
BL3	Omega-3-6-9 (Portugal)	Borage oil; flaxseed oil; salmon oil	1

2.3. FA profile

Fatty acid methyl esters (FAME) were prepared in duplicate by transmethylation using boron trifluoride (Sigma Aldrich St. Louis. MO. USA) according to Shanta & Ackman (1990) with some modifications, and analysed in a Shimadzu GC-2010 gas

chromatograph with a flame ionization detector (Shimadzu, Columbia). The chromatograph was equipped with a CPSil 88 fused silica capillary column (Varian, Middelburg, The Netherlands; 50 m x 0.25 mm internal diameter, 0.19 µm film thickness). He was the carrier gas (120 kPa) and the temperature program was: 120ºC (5 min), 120 - 220 ºC (3ºC/min) and 220 ºC (10 min). Injector and detector temperatures were 250 °C and 270 °C, respectively. The split ratio was 1:50 and the injected volume was 1.0 µl, performed in duplicate assays, each one injected in duplicate. FAME's were identified by comparison with standard mixtures (FAME 37, Supelco, Bellefonte, PA, USA) and data were analysed using GC Solution software (version 2.30, Shimadzu GC solution, Shimadzu, Columbia). Each FA was expressed as relative percentage of the individual FAMEs represented in the chromatogram (Casal & Oliveira, 2007).

2.4. Vitamin E determination

Aliquots of the pooled oil were accurately weighed into microcentrifuge tubes. Then, 20 µl of the internal standard solution (tocol, 1mg/ml) was added and the volume made up to 1 ml with *n*-hexane. After vortex mixed and centrifuged (5 min., 13 000 rpm), the supernatant was transferred to amber glass vials and directly injected into the HPLC system.

The chromatographic analysis was carried out in an HPLC integrated system equipped with an AS-950 automated injector, a PU-980 pump, an MD-910 multiwavelength diode array detector (DAD) and an FP-920 fluorescence detector (Jasco, Japan), programmed for excitation at 290 nm and emission at 330 nm. Normal-phase chromatography was run in a Supelcosil^{TM} LC-SI column (3 µm; 75 x 3.0 mm; Supelco, Bellefonte, PA, USA), operating at room temperature (21 °C), according to Alves et al. (2009).

2.5. Statistical analysis

Using the statistical program IBM SPSS STATISTICS (21.0 package, IBM Corporation, New York, USA), all experimental data were assessed for normality and homogeneity of variances by Kolmogorov-Smirnov and Leven's tests. The results were then analysed using an One-sample T-test to compare the claimed and measured values of oil, EPA, DHA, ALA, EPA+DHA and vitamin E. Significant differences were considered when $p<0.05$.

3. Results

Table 2 shows the FA profile of the sixteen food supplements analysed. Table 3 allows the comparison between the label information provided by the manufacturers and the obtained results (oil, EPA, DHA, ALA and vitamin E contents, mg per one capsule). There were significant differences (p<0.05) between the label information supplied and the experimental data in all analysed nutrients (Table 3)

3.1. Fish oil content

For each sample, the oil content of six individual capsules was calculated as the weight difference between the filled and the empty capsules. The oil content ranged from 472 to 1179 mg per capsule. According with the manufacturer information the majority of the preparations (11 supplements) contained less oil than the label states, but only four had significantly lower values (p<0.05). The other five supplements held more oil than labelled, but only in two samples the oil contents were significantly higher.

3.2. Fatty Acid Composition

The FA analysis of the supplements allowed the identification of 29 individual FAMES, though only 20 were considered significant in terms of weight percent (Tables 2 and 3). As expected, PUFA were the predominant class in all samples, ranging from 31.7 to 85.8 % of total FA. The second most representative group was SFA, with contents varying between 5.0 % and 38.7 %. In general, myristic (14:0), palmitic (16:0) and stearic (18:0) were the most prevalent SFA. The majority of the samples presented palmitoleic (16:1 *n*-7) and oleic (18:1 *n*-9) acids as the main MUFA and their sum varied from 2.2 to 23.7 %. As shown in Table 2, EPA and DHA were the major *n*-3 PUFA in all samples, except in BL1 and BL2, where ALA was found at higher concentrations.

It was verified that: EPA contents ranged from 53.1 to 568.8 mg/capsule; DHA contents ranged from 35.7 to 292 mg/capsule; and ALA contents ranged from 3.3 to 186.4 mg/capsule.

FO6 and BL2 samples contained the higher and the lower total amounts of LC *n*-3 PUFA per capsule (752 and 89 mg/capsule, respectively).

Table 2. Fatty acid profiles of selected omega-3 supplements. Values represent mean ± standard deviation.

Fatty acid (%)	Sample							
	FO1	FO2	FO3	FO4	FO5	FO6	FO7	FO8
14:0	6.89±0.17	0.07±0.00	0.12±0.00	6.28±0.07	5.68±0.44	0.27±0.04	8.80±0.36	-
16:0	15.16±0.47	0.08±0.01	2.78±0.11	17.33±1.97	13.97±0.94	0.52±0.06	18.51±1.40	0.59±0.12
18:0	3.27±0.14	0.32±0.03	3.45±0.38	3.44±0.09	2.78±0.00	0.66±0.10	3.24±0.01	2.21±0.42
20:0	0.39±0.01	0.30±0.01	0.64±0.06	0.66±0.04	0.28±0.03	0.69±0.10	0.55±0.02	0.33±0.02
21:0	2.92±0.15	2.53±0.10	1.60±0.07	3.55±0.19	2.31±0.00	0.89±0.09	2.83±0.00	1.72±0.12
23:0	0.88±0.07	1.90±0.05	1.35±0.22	1.17±0.20	0.77±0.01	1.93±0.06	0.68±0.00	1.63±0.08
∑ SFA [1]	31.28±0.08	5.45±0.17	10.29±0.85	33.30±2.23	27.07±1.50	4.95±0.33	35.87±1.68	6.96±0.67
16:1 *n*-7	8.31±0.02	0.49±0.02	1.32±0.10	8.21±0.09	7.77±0.60	0.42±0.07	9.86±0.17	0.39±0.04
17:1	1.06±0.00	0.14±0.01	0.11±0.00	0.93±0.04	0.85±0.06	0.07±0.01	1.20±0.00	0.18±0.01
18:1 *n*-9	7.94±0.16	1.81±0.05	7.39±0.44	8.54±0.42	11.16±0.02	1.16±0.08	8.87±0.02	9.29±0.70
18:1 *n*-7	3.31±0.05	0.59±0.04	2.66±0.25	2.80±0.19	3.59±0.01	0.36±0.06	3.01±0.03	2.46±0.18
∑ MUFA [2]	19.50±0.16	2.66±0.09	9.93±0.43	19.81±0.37	22.06±0.65	2.15±0.18	22.25±0.15	9.90±0.61
18:2 *n*-6	4.24±0.15	1.48±0.03	2.00±0.06	3.14±0.71	2.97±0.22	0.48±0.07	3.73±0.03	1.50±0.12
18:3 *n*-6	0.94±0.04	0.20±0.02	0.88±0.05	0.93±0.02	1.53±0.18	0.60±0.01	0.14±0.01	0.87±0.04
18:3 *n*-3 (ALA)	0.76±0.01	0.69±0.02	1.73±0.16	1.47±0.05	4.99±0.19	2.41±0.28	0.65±0.01	2.17±0.08
20:3 *n*-6	0.60±0.03	0.40±0.02	2.31±0.21	0.96±0.00	4.94±0.33	2.24±0.19	0.18±0.06	0.74±0.10
20:4 *n*-6	1.26±0.13	3.48±0.09	2.03±0.07	1.20±0.11	1.00±0.11	2.97±0.26	1.26±0.10	3.25±0.03
20:5 *n*-3 (EPA)	18.69±0.17	60.48±0.16	34.43±2.37	19.08±0.23	13.99±0.40	48.15±2.13	15.89±0.12	33.79±0.13
22:4 *n*-6	0.75±0.03	1.84±0.02	1.77±0.05	0.74±0.02	0.58±0.03	1.85±0.10	0.70±0.02	1.29±0.08
22:5 *n*-6	0.36±0.02	0.51±0.00	0.65±0.16	-	0.35±0.02	0.71±0.07	0.41±0.01	2.02±0.68
22:5 *n*-3	2.21±0.08	3.32±0.12	5.26±0.51	2.13±0.29	1.67±0.12	4.17±0.24	1.42±0.11	2.98±0.21
22:6 *n*-3 (DHA)	12.10±0.77	13.04±0.01	19.90±2.02	12.39±0.45	11.93±0.36	18.79±0.63	12.12±0.23	23.78±1.88
∑ PUFA [3]	42.19±1.01	85.81±0.10	70.96±5.53	42.04±0.13	44.13±1.13	82.93±4.00	36.70±0.15	72.74±1.47
∑ *n*-3 PUFA	33.76±1.01	77.53±0.01	61.32±5.06	35.07±0.91	32.58±1.07	73.51±3.28	30.08±0.45	62.71±2.04
∑ *n*-6 PUFA	8.14±0.00	7.91±0.11	9.64±0.47	6.97±0.78	11.38±0.10	8.86±0.69	6.42±0.21	9.67±0.59

Table 2. (continuation)

Fatty acid (%)	Sample							
	FO9	FO10	FO11	FO12	KO	BL1	BL2	BL3
14:0	8.39±0.63	7.75±0.36	3.32±0.01	0.31±0.00	10.35±0.34	3.28±0.05	2.22±0.32	-
16:0	16.89±0.12	17.27±0.43	14.33±0.28	2.04±0.00	19.93±0.07	10.26±0.03	9.11±0.32	14.31±0.41
18:0	3.27±0.03	3.20±0.02	3.26±0.06	3.83±0.03	1.23±0.04	3.43±0.07	2.86±0.13	3.03±0.08
20:0	0.49±0.11	0.42±0.02	0.53±0.02	0.41±0.01	0.17±0.00	0.34±0.01	3.28±0.02	0.51±0.00
21:0	2.70±0.08	3.18±0.01	2.51±0.04	2.56±0.00	3.47±0.04	1.17±0.03	0.80±0.02	2.47±0.00
23:0	0.77±0.05	0.73±0.07	1.20±0.22	1.68±0.00	0.36±0.00	0.34±0.01	0.27±0.00	0.70±0.06
∑ SFA [1]	33.62±0.81	33.92±0.77	26.17±0.66	11.29±0.06	38.69±0.39	19.31±0.16	19.08±0.62	25.57±0.60
16:1 *n*-7	9.70±0.59	8.64±0.27	6.18±0.05	0.75±0.00	5.66±0.16	4.37±0.03	2.78±0.20	6.62±0.26
17:1	1.22±005	1.24±0.00	0.94±0.01	0.10±0.00	0.67±0.01	0.51±0.06	0.34±0.02	1.04±0.04
18:1 *n*-9	8.32±0.24	8.60±0.09	9.49±0.17	7.61±0.02	10.32±0.22	15.98±0.16	11.10±0.22	8.93±0.15
18:1 *n*-7	3.24±0.01	2.92±0.06	3.02±0.05	2.81±0.01	5.66±0.05	1.57±0.14	1.52±0.05	2.94±0.06
∑ MUFA [2]	21.42±0.26	20.86±0.40	19.32±0.23	12.14±0.03	23.69±0.43	21.56±0.05	14.78±0.02	19.63±0.47
18:2 *n*-6	3.65±0.04	3.83±0.07	3.26±0.07	1.04±0.00	3.00±0.07	24.03±0.95	30.85±0.00	3.43±0.05
18:3 *n*-6	0.84±0.01	0.75±0.03	1.21±0.10	0.17±0.00	0.23±0.00	-	-	1.11±0.03
18:3 *n*-3 (ALA)	1.03±0.05	0.49±0.00	1.63±0.02	1.01±0.02	0.60±0.01	19.28±0.54	20.53±0.29	1.46±0.03
20:3 *n*-6	0.51±0.03	0.16±0.01	0.11±0.03	0.20±0.00	0.20±0.00	0.26±0.02	0.25±0.05	1.80±0.09
20:4 *n*-6	1.41±0.12	1.05±0.04	2.61±0.59	2.31±0.01	0.35±0.01	0.42±0.09	0.42±0.06	1.55±0.15
20:5 *n*-3 (EPA)	18.51±0.91	18.18±0.12	18.09±0.06	35.29±0.09	18.15±0.41	6.76±0.04	6.24±0.12	18.30±0.33
22:4 *n*-6	0.68±0.02	0.77±0.00	0.79±0.04	-	-	0.31±0.00	0.24±0.01	0.83±0.03
22:5 *n*-6	0.38±0.02	0.40±0.03	0.49±0.00	-	-	0.14±0.04	0.14±0.02	0.51±0.00
22:5 *n*-3	1.83±0.07	1.94±0.08	2.30±0.03	4.44±0.01	0.43±0.01	0.69±0.06	0.80±0.09	2.50±0.01
22:6 *n*-3 (DHA)	10.75±0.27	12.12±0.26	13.42±0.25	24.77±0.02	8.74±0.14	4.55±0.18	4.19±0.28	13.07±0.09
∑ PUFA [3]	39.78±0.51	39.90±0.46	44.07±0.92	69.87±0.07	31.71±0.50	56.44±1.05	63.67±0.33	44.80±0.05

The experimental EPA levels differ significantly from the ones given on the labels, except for samples FO2 and FO3. The differences verified ranged from 18% lower in FO7 to 71% higher in KO sample. Fourteen samples contained either equal or significantly higher levels of DHA compared to the label information, with sample FO6 containing almost 3 times the stated amount (211 mg vs. 75 mg, respectively). Samples FO10 and FO11 did not declare the amount of *n*-3 PUFA, although sample FO11 stated EPA+DHA content. Brands BL1, BL2, and BL3 are blends of fish oil and vegetable oils, formulated to supply omegas 3, 6 and 9. Samples BL1 and BL2 presented significantly higher ALA levels than claimed (24 and 5%, respectively) while BL3 contained significantly less ALA than the labelled amount (14.5 vs. 200 mg/capsule). FA profile of this sample (BL3) is very similar to the fish oil profile, indicating that, contrary to what is claimed, flaxseed oil is absent or in very low amounts. FO2, FO6 and FO12 samples contained higher EPA and DHA levels than the other samples, which is in accordance to the presence of fish oil concentrate referred on the label.

3.3. Recommended Daily Doses

Current dietary recommendations for EPA and DHA are based on cardiovascular risk considerations and range between 250 and 500 mg/day for healthy adults (EFSA, 2010; Kris-Etherton et al., 2009). For individuals with established cardiovascular disease (CVD), the American Heart Association (AHA) recommends the intake of 1 g/day of EPA and DHA (Smith et al., 2006). Although recommended doses may be achieved by consuming two servings of (preferably oily) fish per week, supplementation of the diet with oil supplements can be an alternative to ensure an optimal *n*-3 PUFA intake (Smith et al., 2006).

Based on the recommended daily doses (RDD) from manufacturers, sample BL2 fail to meet minimum concentration of LC *n*-3 PUFA recommended for primary prevention of coronary disease (250 mg/day), even when using the highest recommended dosage (± 180 mg/ 2 capsules). The ingestion of the RDD of FO5, FO9, KO, BL1, and BL3 samples provides less than 500 mg/day of EPA+DHA, with levels ranging from 287 to 328 mg/day. For all the other samples, the RDD exceeded the 500mg/day. FO3, FO6, and FO12 samples provided more than 1 g/day of total LC *n*-3 PUFA, the recommended daily intake by AHA for individuals with established CVD, with sample FO12 delivering almost three times that dose (2832.0 mg EPA and DHA).

Table 3. Oil, EPA, DHA, ALA, EPA+DHA and Vitamin E contents in tested omega-3 supplements (label information vs. experimental).

Sample	Contents in 1 capsule – label claims (mg)						Contents in 1 capsule – dete			
	Oil	EPA	DHA	ALA	EPA+ DHA	Vitamin E	Oil	EPA	DHA	
FO1	500	90	60	ND	150	2.5	471.83±20.89	88.18±0.94*	57.08±3.07	3.
FO2	1018	580	120	ND	800	4.5	940.43±25.60	568.75±22.60	122.65±0.25*	6.
FO3	500	165	110	ND	315	ND	506.23±7.11	174.28±9.78	100.76±8.34	8.
FO4	500	90	60	ND	150	7.5	510.30±7.80	97.39±1.02*	63.21±2.44	7.
FO5	550	58	53	ND	111	11	550.80±10.02	77.08±1.82*	65.71±1.77*	27
FO6	1000	500	75	ND	575	6.7	1123.40±4.17*	540.92±19.74*	211.04±6.22*	27
FO7	1000	180	120	ND	300	0.7	933.97±34.60	148.36±8.97*	113.18±17.65	6.
FO8	1000	350	250	ND	600	7.5	989.30±28.93	334.27±4.33*	235.23±15.20	21
FO9	1000	160	100	ND	260	ND	980.70±5.38*	181.49±9.57*	105.39±14.62	10
FO10	1000	ND	ND	ND	ND	10	952.90±13.18	173.23±2.83	115.50±4.99	4.
FO11	1000	ND	ND	ND	230	ND	992.17±0.92	179.53±4.50	133.13±3.38	16
FO12	1200	360	240	ND	600	ND	1178.83±16.00	415.97±0.98*	292.01±0.59*	11

3.4. Vitamin E Analysis

The determined levels of vitamin E are presented in Table 3. For most of the samples (except supplement FO4), total vitamin E content was significantly different (p<0.05) from the label claims. Vitamin E levels were not stated on the label of FO3 and FO11 samples, and FO9 and FO12 samples refer the presence of vitamin E but not its levels. Vitamin E contents ranged from 0.18 mg/capsule (FO11) to 19.1 mg/capsule (KO). Considering the manufacturers RDD, almost half of the analysed products provided 100% or more of the RDA for vitamin E (15 mg/day), with KO and FO3 providing almost 3 times the RDA (255 and 275%, respectively). The required amount of vitamin E and the recommended intake depends largely on the level intake of PUFAs (Horwitt, 1986). Vitamin E is added to *n*-3 supplements mainly for preservation purposes as an antioxidant but, additionally, may also contribute to neutralize the oxidative damage resulting from the consumption of those *n*-3 PUFAs supplements (Meydani, 1992).

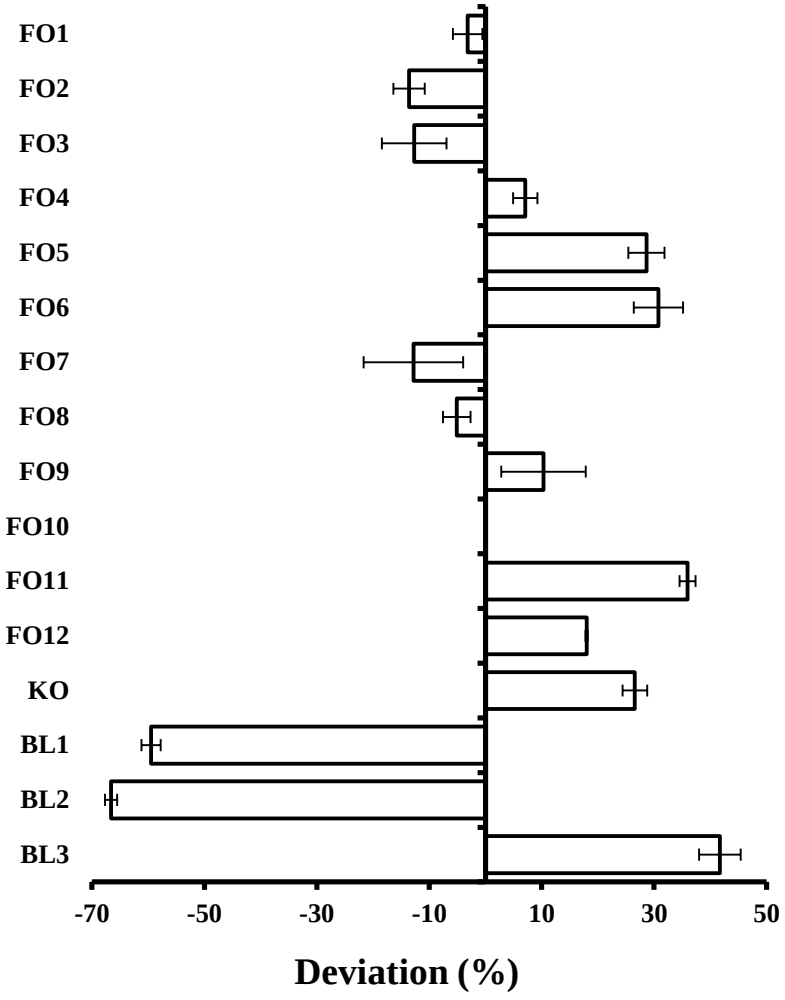

Figure 1. EPA+DHA deviation (%) from label information. Bars represent mean ± standard deviation. ND - No Data Available.

4. Discussion

In the last two decades, *n*-3 food supplements have become very popular because of the wide range of proposed health benefits, including prevention of cardiovascular disease. This type of supplements are generally considered as safe, free of detectable traces of mercury (Foran et al., 2003), with low levels of polychlorinated biphenyls (PCBs) (Melanson et al., & Lewandrowski, 2003) and, as a result of the technology improvement, their quality is increasing. Nevertheless, there have been reports that some products failed to meet quality guidelines, namely regarding label accuracy on *n*-3 PUFA contents.

Our results revealed significant differences between the amounts of oil, EPA, DHA, and ALA displayed on the label and experimentally obtained values. In Table 3 and Figure 1, the same trend can be noticed, since most of the samples have significant higher or lower levels ($p<0.05$) of EPA+DHA than the manufacturer's information stated. The most critical deviations are verified in blends of fish and vegetable oils (BL1: - 60%; BL2: - 67% and BL3: 42%). Also, there was a great variability in the content of EPA and DHA as well as in the recommended daily intake. Consumption of the same amounts of *n*-3 supplements may result in differences of up to 35.2% in LC *n*-3 PUFAs. These discrepancies have been reported in previous studies. Tatarczyk et al. (2007) analysed nine different commercial fish oil supplements available in the Austrian market for their fatty acid content. They found label claims for EPA and DHA contents reasonably accurate for the majority of preparations. They also denoted a high variability in EPA and DHA contents among products, a similar outcome to what was found in the present study. In 2010, Kolanowski found that the LC *n*-3 PUFA concentration of nineteen brands of fish body oil and fish liver oil capsules purchased in Poland market was similar to the concentration given by the manufacturer for the majority of the products (Kolanowski, 2010). More recently, the study of 45 fish oil supplements available in the South Africa market has revealed that more than half of the *n*-3 fatty preparations did not contain the claimed EPA and/or DHA contents as stated on product labels (Opperman et al., 2011).

5. Conclusions

Omega-3 supplements are generally considered an effective and safe alternative for individuals with low fish consumption to ensure an adequate dietary level of LC *n*-3 PUFA, thus preventing the risk of many diseases. Considering their widespread use it is of paramount importance that these products are labelled accurately and unambiguously, thus providing guidance for consumers to make informed decisions concerning their

purchase. Our results and the previous outcomes reveal the need for practical intervention and improvement of label information quality. To enhance the overall quality of omega-3 supplements it is urgent to implement more effective quality control of the processes, including raw materials, in-process and finished products, in order to ensure better information for the consumers and health professionals of the products available on the market.

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2.3– Suplementos Alimentares com Fitoestrogénios

E. Food supplements intended for menopause symptoms relief: isoflavones levels and bioavailability using Caco-2 cell model. *submitted*

Food Chemistry

submitted

Food supplements intended for menopause symptoms relief: isoflavones levels and bioavailability using Caco-2 cell model

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Abstract

A simple and efficient matrix solid-phase dispersion (MSPD) method was optimized and validated for the extraction of native isoflavones from food supplements (n=15) intended for menopause. The compounds were analysed RP-HPLC-DAD. The proposed method was successfully applied to the analysis of puerarin, daidzin and genistin, daidzein, glycitein, genistein, formononetin, prunetin, and biochanin A. Recoveries ranged from 90.1% to 101.2% for all isoflavones, and relative standard deviations were below 6%. Isoflavones were quantified and the results compared to the labelled information. Label claims were inconsistent with the determined isoflavones content for the majority of the assayed samples. Only four food supplements comply with the recommendations made by the European Community on the tolerable thresholds of the content of claimed constituent (80–145%). Isoflavones bioavailability of three food supplements (standardized isoflavonoids from soy, soy extract, and red clover) was further investigated using human intestinal epithelial Caco-2 cell monolayers. The apparent permeability coefficients (Papp) of selected isoflavonoids across the Caco-2 cell monolayers were found to be affected by the isoflavone concentration and the product matrix.

Keywords: Food supplements, menopause, isoflavones, HPLC-DAD, bioavailability, Caco-2 cells

Introduction

Today consumers are interested in taking an active role in their health and self-care, by selecting specific foods and other items, including food supplements, that they believe it can improve their health and quality of life. Food supplements are generally used to overcome nutritional deficiencies, prevent or reduce the risk of disease, and/or to promote general well-being. The wide and steadily growing consumption range and popularity of food supplements, constitutes a challenge for consumer protection. Food supplements are not subjected to standardized quality control measures, unlike pharmaceuticals, and, therefore, the presence of impurities, adulteration, and dosage inconsistency are more frequent. Several studies have shown a great variability in marketed products regarding the concentration and source of isoflavones, stressing the need of standardization and quality control of these products, considering its therapeutic use (Boniglia, Carratù, Gargiulo, Giammarioli, Mosca & Sanzini, 2009). Additionally, the biological effectiveness of these bioactive compounds depends greatly on the intestinal bioavailability, variable between isoflavones.

Isoflavones are phytoestrogens, plant-derived compounds that have weak estrogenic activity. Their structural analogy to the estradiol molecule confers them hormonal effects, including the ability of binding to estrogen receptors and modulating hormone-dependent processes (Tham, Gardner & Haskell, 1998). Isoflavones are primarily found in plants of the Fabaceae family, including soy, lentils, bean plant, chickpeas, alfalfa and red clover (Fletcher, 2003). A number of epidemiological studies have linked the consumption of isoflavone-rich foods with low incidence of the major hormone-dependent cancers (Adlercreutz, 1995; Severson Nomura, Grove & Stemmerman, 1989), cardiovascular diseases (Clarks, Anthony & Hughes, 1995), osteoporosis (Tham et al., 1998), and climacteric complaints (Adlercreutz, Hämäläinen, Gorbach & Goldin, 1992).

Driven by these purported health benefits, a plethora of products containing isoflavones have come on to the market, specifically targeting women in menopause. These preparations generally contain extracts from soy, red clover and kudzu, as single ingredients or in multi-ingredient formulations mixed with minerals, vitamins, other plant extracts, omega-3, 6, and 9 fatty acids, etc.

The three major isoflavones found in soybean (Glycine max (L.) Merril) are daidzein, glycitein and genistein, which occur mainly as glycoside, acetylglycoside, and malonylglycoside forms. In soy derivates, including food supplements, the glycoside and aglycone forms are the main components (Coward, Smith, Kirk & Barnes, 1998). In

contrast to soybean, red clover (Trifolium pratense L.) contains numerous isoflavones, being biochanin A and formononetin, and their glycosides and malonyl derivatives, the major isoflavonoids components (Rijke, Zafra-Gómez, Ariese, Brinkman & Gooijer, 2001; Wu, Wang, & Simon, 2003). Pueraria mirifica Airy Shaw et Suvatabhandu (Fabaceae), commonly known as White Kwao Krua and Thai kudzu, is an indigenous herb to Thailand, and has traditionally been used as folk medicine for rejuvenation and to attenuate menopausal symptoms. The dried powder of the plant tubers has also been used to prepare food supplements. Several isoflavonoids have been identified in P. mirifica tubers, including daidzin, puerarin, daidzein, genistin, and genistein (Malaivijitnond, 2012).

Isoflavones, like the majority of polyphenols, are usually found in plants mainly as glycosides and glycoside esters (Tsao, 2010). After ingestion they are metabolised by bacteria in the gastrointestinal tract, releasing their aglycones, the truly bioactive constituents (Day, et al., 1998; Setchell et al., 2002). It is, therefore, very important to know the bioavailability of such compounds in order to understandand their biological activity. In this perspective, the Caco-2 cell line, derived from human colorectal adenocarcinoma, has been widely used as an in vitro model of the human small intestine for predicting drug intestinal absorption and excretion in humans (Artursson, Palm & Luthman, 2012). Several Caco-2 cell line studies have examined the intestinal absorption of isoflavones as pure compounds (Steensma, Noteborn, van der Jagt, Polman, Mengelers & Kuiper, 1999; Murota, Shimizu, Miyamoto, Izumi, Obata, Kikuchi & Terao, 2002; Chen, Lin & Hu, 2005; Tian, Yang, Yang & Wang, 2009), but studies using isoflavones-containing food supplements extracts are still very scarce (Wang, Chen, Joseph & Hu, 2008).

The aim of the present study was to optimize and validate a simple analytical procedure for the quantification of nine isoflavones (puerarin, daidzin and genistin, daidzein, glycitein, genistein, formononetin, prunetin, and biochanin A) in 15 commercial food supplements, by Matrix Solid-Phase Dispersion (MSPD) and reversed-phase (RP) HPLC-DAD. The obtained quantitative data were compared with the labelled contents. Moreover, a study was undertaken to measure permeability in the human colon adenocarcinoma Caco-2 cell line of selected isoflavones from selected food supplements.

2. Material and methods

2.1 Chemicals and reagents

Puerarin (≥99%), daidzin (≥95%), genistin (≥95%), glycitein (≥97%), daidzein (≥98%), genistein (≥98%), biochanin A (≥97%), prunetin (≥98%), and formononetin

(≥99%) and the internal standard 2-methoxyflavone were purchased from Sigma-Aldrich (St. Louis, MO, USA). Preparative C18 sorbent (125 Å, 55-105 µm) was from Waters (Milford, MA, USA). Water was purified with a “Seradest LFM 20” system (Seral, Ransbach-Baumbach, Germany). The eluents were filtered through 0.45 µm filters and degassed under reduced pressure. Disposable cellulose acetated 0.45 µm was from OlimPeak, Teknokroma (Barcelona, Spain). HPLC grade solvents, methanol and acetonitrile, and analytical grade formic acid were from Merck (Darmstadt, Germany).

Caco-2 (ATCC HTB-37) human colon adenocarcinoma cell line was purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). Dulbecco’s Modified Eagle’s Medium (DMEM) with 4.5 g/L glucose and GlutaMAX™, fetal bovine serum (FBS), 0.05% trypsin–EDTA, penicillin–streptomycin (Lonza Biowhittaker, Verviers, Belgium) and non-essential amino acids (NEAA) were obtained from Gibco (Life Technologies, Paisley, UK). Phosphate buffer saline (PBS) was purchased from Sigma Chemical Co., USA. Tissue-treated inserts (high density PET membrane, 23.1 mm, 0.3 µm pore size, 4.2 cm2) were from Becton Dickinson Falcon™ (Bedford, MA, USA) and 6 wells plates were purchased from Orange Scientific (Braine-l'Alleud, Belgium).

2.2 Standards

Purity-corrected individual isoflavones stock solutions were prepared in DMSO (1 g/L) and then serially diluted in water: methanol (10:90, v/v) in order to obtain the standard concentrations for the calibration curves. A working 2 mg/L solution of the internal standard was also prepared in DMSO. All solutions were stored in amber glass vials at 4 °C.

2.3 Samples and sample preparation

2.3.1 Sampling

Fifteen different food supplements containing isoflavones were purchased from local retail, herbal stores and a pharmacy. Their selection was based on availability on the market, trying to include a broad range of isoflavone profiles and concentrations. Table 1 shows the composition of the food supplements and the recommended daily dose (RDD) as indicated by the manufacturer. The food supplements were in the form of tablets and capsules. Ten tablets or capsules were taken from each product. Tablets were ground to a fine powder using a glass mortar and pestle, after carefully removing the colour coatings, if present. Shells from hard-gelatine capsules were removed and the content

mixed with mortar and pestle. Contents of soft gel capsules were squeezed into a test tube and mixed.

Table 1. Sample code and specifications of the analysed food supplements as provided by the manufacturers.

Sample Code	Country of origin and composition	Dosage form	Capsule/ tablet weight (g)
S1	EU. Natural isoflavonoids from non-transgenic **soy**	Capsule	0.394
S2	France. **Soy extract** (*Glycine max*); excipients	Capsule	0.334
S3	France. Tomato extract, milk proteins, **soy extract**, vitamin C	Capsule	0.740
S4	United Kingdom. Evening primrose oil; s**oy isoflavones**; fish oil; vitamin E, excipients	Gel capsule	0.564
S5	UE. Coral Calcium, **soy extract** rich in phytoestrogens, Passiflora; excipients	Tablet	1.237
S6	France. Yam extract (*Dioscorea opposita* Thunb), **soy extract** (*Glycine max*), fructo Oligo Saccharides, hop (*Humulus lupulus*), meadowsweet (*Spiraea ulmaria*) , grape vine (*Vitis vinifera*), vitex (*Vitex agnus castus*); vitamin E; selenized yeast	Capsule	0.307
S7	Portugal. *Discorea opposite*, wild yam, **Soy** (*Glycine max*) (pure isoflavones), primrose oil, Dong Quai (A*ngelica sinenis*); melissa *(Melissa officinalis*); sage *(Salvia officinalis*), siberian ginseng (*Eleutherococcus senticosus*); hop (*Humulus lupulus);* vitex *(Vitex agnus-castus);* vitamins E, B6	Gel capsule	0.470
S8	Spain. Red algae (*Lithothamnium calcareum)*; **fermented soy; soy isoflavones**	Capsule	0.565
S9	EU. Evening primrose oil (*Oenothera biennis)*; **soy isoflavones**; vitamin E; excipients	Gel capsule	0.447
S10	Belgium. Sage extract; **soy extract**; saffron (*Crocus sativus*); vitamin B6	Tablet	0.772
S11	EU. **Soy isoflavones;** sage; oat; marine magnesium; vitamin E; excipients	Capsule	0.538
S12	Italy. **Soy isoflavones** (with 55-72% genistin/genistein); excipients	Capsule	0.623
RC	Australia. Standardized **red clover** (*Trifolium pratense*) extract; excipients	Tablet	0.351
SR	Spain. **Soy rich in isoflavones** of retarded action; yam extract; **red clover extract**; vitamins A, C, B1, B2, B12, E, excipients	Capsule	0.634
TK	USA. Vitamin B12; **standardized *Pueraria mirifica* root extract (Thai Kudzu)**: miroestrol, isoflavonoids; pyridoxal-5 phosphate; biotin; folic acid; excipients	Capsule	0.519

2.3.2. Matrix solid-phase dispersion

An aliquot of 0.5 g of the previously homogenized samples, 2 g of C18 and 0.5 mL of the internal standard (2 g/L,) were placed in a glass mortar and blended together using a glass pestle to obtain a complete disruption and dispersion of the sample on the solid support. After blending was complete (1min), the sample was packed into an empty column containing a polyethylene frit at the bottom. A second frit was placed on the top of the sample by careful compression with a syringe plunger. The packed column was attached to a vacuum manifold (Visiprep, Supelco) coupled with a vacuum pump and the flow adjusted to 1mL/min. The column was rinsed with 10 mL water (discharged fraction) and the elution step was carried out with 2×5mL of water: methanol (10:90, v/v) (collected fraction). The extracts collected in amber vials were filtered through disposable 0.45 µm cellulose membranes before HPLC analysis. Sample extraction was performed in triplicate.

2.4. HPLC equipment

The chromatographic analysis was performed using an HPLC unit (Jasco, Japan), consisting of two Jasco PU-2080 Plus HPLC pumps, an AS-950 automated injector (20 µL loop), and a MD-2010 Plus multiwavelength diode-array detector (DAD). The separation of the isoflavones was carried out on a reversed-phase Luna C18 column (4.60 mm × 150 mm, 5 µm particle size) from Teknokroma (Barcelona, Spain), maintained at 40 °C. The mobile phase consisted of 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B). The gradient program was previously developed in our laboratory (Visnevschi-Necrasov et al., 2009), and was used with some modifications: 0 min 0% B, 15min 32% B, 18 min 45% B, 23 min 50% B, 25 min 70% B, 35 min 10% B, maintaining these conditions for 5 min and returning to the initial conditions within 3 min. The flow rate of the mobile phase was 1 mL/min and the injection volume was 20 µL.

UV spectral characteristics and peaks purity were evaluated trough DAD. Peak purity measurements of all compounds were based on spectral comparison at three different peak heights. Analytes were monitored at 254 nm and quantification was performed on the basis of the internal standard method. Chromatographic data were processed with ChromNAV Software (Jasco, Japan).

2.5 Caco-2 cell culture

The human colorectal carcinoma cell line Caco-2 was obtained from ATCC. Cells were routinely cultured in 75-cm2 flasks in Dulbecco's modified Eagle's medium (DMEM) containing D-glucose (4.5 g/L) and GlutaMAX™ and supplemented with 10% fetal bovine serum (FBS), 1% penicillin and streptomycin (10,000 U/mL), and 1% MEM Amino Acids. The cells were maintained in a humidified atmosphere of 5% CO_2/95% air at 37°C, and were supplied with fresh medium every 2 days. Cells were subcultured at 80-90% confluence. For experiments, Caco-2 cells were seeded in cell culture inserts in 6-well plates at a density of 4×104 cells/cm^2. The basolateral and apical compartments contained 2.5 and 1.5 mL of culture medium DMEM, respectively. Culture medium was replaced every other day. The integrity of the Caco-2 cell monolayer was checked by Transepithelial Electrical Resistance (TEER) measurements using an epithelial voltammeter (EVOM, World Precision Instrument, Sarasota, FL, USA) for the whole period.

2.6 Isoflavones permeation experiments

Cells used for the current study were from passage 65. Experiments in triplicate were performed 21 days after seeding. The cell monolayers were pre-equilibrated with fresh PBS pH 7.4 previously warmed at 37 °C, for 30 min, and the incubation medium was then discarded. Afterwards, 1.5 mL of the test solutions, prepared by dilution of the MSPD extracts in PBS, were added to the apical side of the Caco-2 monolayers and 2.5 mL of PBS to the basolateral side, and allowed to permeate for 120 min at 37 °C under 5% CO_2, 95% of relative humidity. Samples (0.5 mL) were withdrawn from the receptor side at 0, 15, 30, 60, and 120 minutes for the determination of the isoflavone transported across the monolayer. After each sampling, the basolateral side was replenished with the same PBS volume. Samples were preserved at -20 °C for subsequent HPLC analysis as described in section 2.4.

The apparent permeability to the isoflavones, expressed in cm/sec, was calculated from the following equation: Papp = Q/ (A x C x t) where, Q is the total amount of permeated isoflavones during the 120 minutes of experiment (µg), A is the diffusion area (cm2), C is the donor compartment concentration at time zero (µg/mL), and t is the time of experiment (s). In addition to Papp, the percentage of permeation (%) of the isoflavones daidzein, genistein, formononetin, and biochanin A was calculated as the proportion of the

original amount that permeated through the monolayer, which was calculated as the amount transported divided by the initial amount in the apical chamber.

3. Results and Discussion

Commonly used methods for extraction of native isoflavones are based on solvent extraction using polar organic solvents such as methanol, ethanol, acetonitrile and acetone, water diluted or not, followed or not by a clean-up step to eliminate interfering compounds. In this case, isoflavones are analysed in their various conjugated forms or as the aglycones, depending upon the objectives of the research. When determining the total aglycone content, hydrolysis using acid, alkaline or enzymes is used to release isoflavones from their sugar moieties (Rostagno, Villares, Guillamón, García-Lafuente & Martínez, 2009; Shao, Duncan, Yang, Marcone, Rajcan & Tsao, 2011). When a hydrolysis step is employed, the unstable malonyl and acetyl derivatives are converted into more stable forms such as β-glycosides and/or aglycones. At the same time, the chromatographic complexity is reduced. On the other hand, a possible drawback is the increase of the analysis time and/or the possible degradation of compounds (Alves and Oliveira, 2013). Knowledge of the original isoflavone profile of food supplements is important, since their therapeutic potential seems to depend on the levels and the composition of the isoflavones present (Stechell et al., 2001; Rufer, Bub, Möseneder, Winterhalter, Sturtz & Kulling, 2008). The use of Matrix Solid-Phase Dispersion (MSPD) for sample preparation is a recent and increasing approach due to the flexibility and versatility of the process, which allows sample extraction and clean-up in one single step. MSPD has been used for the extraction of pesticides, pharmaceuticals, and other contaminants (Capriotti, Cavaliere, Giansanti, Gubbiotti, Samperi & Laganà, 2010), but also in the isolation of naturally-occurring compounds from different plants (Ziaková, Brandšteterová & Blahová, 2003; Ryjke, Out, Niessen, Ariese, Gooijer & Brinkman 2006; Dawidowicz & Rado, 2010; Shi et al., 2011) including the extraction of isoflavonoids from leguminous plants (Rijke, Kanter, Ariese, Brinkman & Gooijer, 2004; Xiao, Krucker, Albert & Liang, 2004; Visnevschi-Necrasov, Cunha, Nunes, & Oliveira, 2009).

3.1. Method validation

The MSPD methodology used in this study for isoflavones analysis in the selected food supplements was based on the procedure described by Visnevschi-Necrasov et al. (2009) with several modifications. Method optimization was performed in order to reduce

the use of organic solvents. For extraction, the dichloromethane-methanol mixture (25:75, v/v) was replaced by methanol-water (90:10, v/v), a greener and less toxic solvent than the previous one, also described as adequate for isoflavonoids extraction (Klejdus et al., 2005; Rijke et al., 2004). The modified methodology was, then, validated in order to evaluate its efficiency for isoflavones analysis in food supplements.

The set of standards used was chosen to represent the main isoflavonoids found in soy, red clover, and Thai-kudzu products: puerarin, daidzin, genistin, daidzein, glycitein, genistein, formononetin, prunetin, and biochanin A.

The retention times, linear regression data, limit of detection (LOD) and limit of quantification (LOQ) values of the nine isoflavones investigated are presented in Table 2.

Table 2. Retention times (t_R), linear regression data, LOD and LOQ values of the nine isoflavones investigated.

Isoflavones	t_R (min)	Regression equation[a]	Linear range (mg/mL)	R^2	LOD (ng/g)	LOQ (ng/g)
Puerarin	11.287	y = 29.933x + 0.0217	0.010-0.050	0.9995	23.9	79.8
Daidzin	12.641	y = 23.197x + 0.1485	0.050-0.250	0.9994	138	459
Genistin	14.661	y = 24.982x + 0.1742	0.050-0.250	0.9991	161	535
Daidzein	18.303	y = 35.457x + 0.0103	0.005-0.025	0.9995	12.6	41.8
glycitein	18.712	y = 21.774x + 0.0054	0.005-0.025	0.9994	12.9	43.2
Genistein	20.657	y = 35.871x + 0.0132	0.005-0.025	0.9992	17.9	59.7
Formononetin	22.367	y = 33.257x + 0.0108	0.005-0.025	0.9994	13.9	46.5
Prunetin	25.624	y = 39.776x + 0.0119	0.005-0.025	0.9983	22.6	75.4
Biochanin A	25.799	y = 35.825x + 0.0043	0.005-0.025	0.9982	23.5	78.4

[a]y: standard peak area/internal standard peak area; x: concentration (mg/mL of injected solution).

Standard curves were obtained by plotting standard concentration, at five concentrations (mg/mL of injected solution), as a function of the ratio between the peak areas of each standard and the internal standard. Standards were subjected to the entire extraction method and injected in triplicate. Standard curves were linear over the concentration range tested, with correlation coefficients greater than 0.998 for all the analytes. Relative standard deviations (RSDs) of the triplicate injections varied between 0.4 and 4.1 %. The deviation in the retention time was less than 0.06%. The LOD and the LOQ for each isoflavone were calculated as 3.3 and 10 times the standard deviation of the background noise divided by the slope of the calibration curve, respectively. The obtained LOD values were in the range of 12.6–161 ng/g; LOD values ranged from of 41.8–535

ng/g. For validating analytical accuracy (in terms of recovery), duplicate 0.5 g portions of samples S10 (soy), RC (red clover), and TK (Thai-kudzu) were spiked with 0.1 mg of all isoflavones, and extracted as described. For the three samples, the mean recovery for all isoflavones ranged from 90.1 to 101.2%, whereas calculated relative standard deviations (RSD) were below 6.0% (Table 3). The results indicate that the MSDP extraction method was accurate and precise.

Table 3. Recoveries of the nine isoflavones from soy, red clover, and Thai-kudzu samples. Values are mean ± SD, n=3.

Compound	Soy		Red clover		Kudzu	
	Recovery %	RSD %	Recovery %	RSD %	Recovery %	RSD %
Puerarin	98.1	2.5	94.3	2.8	96.3	1.5
Daidzin	91.1	0.2	90.1	0.9	94.2	0.7
Genistin	92.4	1.7	95.5	1.3	93.6	0.9
Daidzein	92.5	3.2	94.2	2.4	102.0	4.8
glycitein	94.2	1.1	92.6	2.0	93.6	0.6
Genistein	101.2	4.2	97.3	3.5	100.8	5.2
Formononetin	95.0	3.5	96.0	1.7	99.8	0.2
Prunetin	99.2	2.8	100.5	4.2	100.5	5.2
Biochanin A	91.5	4.5	95.7	2.9	93.6	6.0

3.2 Analysis of food supplements

The proposed method was applied to the quantification of 9 isoflavones in fifteen food supplements used in the management of menopause symptoms, in capsules and tablets, locally available, described in Table 1. The determined amounts of the individual isoflavones in the samples (mg/unit), total isoflavones (sum of the individual isoflavones), and the percentage of total isoflavones with respect to the stated content given in the label are presented in Table 4. The total isoflavones content per unit ranged from 4.5 to 110.9 mg. The percentages of isoflavones with respect to the stated content ranged from 42% to 139%, except for S7 and TK, with 9% and 189% of the label claim, respectively. Label claims, in the majority of the assayed samples, were inconsistent with the determined isoflavones content, with only four food supplements (S9, S10, S12, and RC) compliant with the recommendations made by the European Community on the tolerable thresholds of the claimed constituent content (80–145%) (EC, 2012). Nevertheless, it is important to state that only 9 isoflavones (aglycones and glycosides) were quantified, due to the fact that they are major compounds in these type of matrices. Moreover, malonyl and acetyl, as well as other isoflavone derivatives could also be present but were not considered in

this study. For example, very recently, Yanaka, Takebayashi, Matsumoto and Ishimi (2012) identified isoflavone succinyl glucosides in soy-based products. On the other hand, the isoflavones content shown on the labels of all the supplements analysed were unclear, since the isoflavone forms included in the total values was not always specified. Therefore it was sometimes difficult to compare the labelled values with those determined in this experiment.

The isoflavone profile of the food supplements revealed a large variability among products containing soy. The concentrations of individual isoflavones were labelled in four preparations (Table 4). Nevertheless, there were considerable differences between the determined and claimed content for all the individual isoflavones. The relative amounts of the compounds in the food supplements will depend on the isoflavone composition in the primary raw material and tissue of the soybean from which they are derived. The isoflavone contents of the soy germ and the soy extract used in food supplements are different, with germ containing typically at least about three times the isoflavone content of the whole seed. The soy germ is richer in daidzin and glycitin, while in the cotyledons, genistin is the main component (Eldridge and Kwolek, 1983; Kim et al., 2007). Since differences in the biological activity of the individual isoflavones are recognized, the registered variability in the relative amounts of the different isoflavones may have a serious impact on the efficacy of the soy-based supplements.

Soybeans and foods derived from soy are major dietary products for Asian populations, and have been linked to a variety of health outcomes (including low incidence rates of breast and prostate cancers, and reduction of menopause symptoms). Several works have estimated that dietary intake of isoflavones by those populations range of 30-50 mg/day (expressed as aglycone equivalents) (Kikuchi, Shimamura, Hirokado, Yasuda & Nishijima, 2001; Somekawa, Chiguchi, Ishibashi & Aso, 2001; Messina, Nagata & Wu 2006). Therefore, such studies provided the background for many clinical researches about the effects of isoflavones consumption, which have used supplements containing at least 40 mg of isoflavones (Howes, Howes & Knight, 2006; Coon, Pittler & Ernst, 2007; Pendleton et al., 2008; Taku, Melby, Kurzer, Mizuno, Watanabe & Ishimi, 2010). According to the recommended daily doses described in the labels, only five samples (S3, S9, S12, RC, and SR) can provide that daily amount of isoflavones, with samples S9 and S12 supplying two times that level.

Table 2. Isoflavones content in the 15 food supplements analysed (mg/unit) and percentages of total isoflavone with respect to the labelled content.

Product type	Sample code	Individual isoflavones, mg/unit									Total isoflavones[a]	Labell isolavo
		puerarin	daidzin	genistin	daidzein	glycitein	genistein	formononetin	prunetin	biochanin A	mg/unit	mg/u
Soya	S1	nd	0.90	1.22	2.24 (7.75)	0.05 (5.20)	5.28 (2.02)	nd	nd	nd	9.7	15
	S2	nd	5.80	1.30	0.15 (5.5)	0.07	0.01	nd	nd	nd	7.3	17.5
	S3	nd	8.50	10.16	0.48	0.13	0.08	nd	nd	nd	19.4	25
	S4	nd	0.47	0.10	7.66	0.13	16.05	nd	nd	nd	24.4	40
	S5	nd	23.79	5.77	1.68	0.65	1.40	nd	nd	nd	33.3	60
	S6	nd	9.86	2.10	1.21	0.64	0.29	nd	nd	nd	14.1	20
	S7	nd	3.38	0.84	0.13	0.08	0.04	nd	nd	nd	4.5	50
	S8	nd	2.69	3.82	12.97	0.17	5.83	nd	nd	nd	25.5	60
	S9	nd	3.83	5.10	21.70 (12.8)	0.33 (1.4)	9.08 (20.8)	nd	nd	nd	40.0	35
	S10	nd	8.69	18.71	2.10	0.48	2.05	nd	nd	nd	32.0	34
	S11	nd	5.97	2.57	0.76	0.29	0.54	nd	nd	nd	10.1	15
	S12	nd	34.00	69.01	5.37	0.42	2.07	nd	nd	nd	110.9	80
Red	RC	nd	nd	0.048	0.15	0.58	0.83	16.19	nd	26.83	44.6	40

Previous works on the quantification of isoflavones in food supplements have also reported inconsistencies in the isoflavone content from that claimed by the manufacturers. Setchell et al. (2001) analysed 33 supplements containing isoflavones and revealed significant differences between labelled and determined isoflavone contents, with approximately half of the supplements presenting lower isoflavones content than the indicated. Nurmi, Mazur, Heinonen, Kokkonen & Adlercreutz (2002) analysed fifteen soybean-based supplements available in Finland and found only one had the content presented on the label, with the remaining products presenting an isoflavones content lower than claimed. More recently, Clarke, Bailey and Lloyd (2008) studied 35 food supplements available in the UK, Canada, and Italy. In that study, eleven preparations did not contain the claimed content and only 14 food supplements were found to deliver more than or equal to 40 mg/day of aglycone isoflavones. Similar outcomes were reported by Boniglia et al. (2009) after analysing 14 food supplements intended for menopausal symptoms, available in Italy. In this study, soy aglycones were determined after hydrolysis, and it was concluded that in more than 50% of the analysed products isoflavones contents were below those claimed.

3.3 Permeation of food supplement extracts across Caco-2 monolayers

The Caco-2 cell monolayer model was chosen for the investigation of the permeability of daidzein, genistein, formononetin, and biochanin A present in four food supplements S1 (standardized isoflavonoids from soy), S6 (soy extract), and S11 (red clover), and for comparing the absorption between the different supplements. Test solutions were prepared to contain 1 mg/mL of their respective product. Permeation of the selected isoflavones present in the food supplements, through the Caco-2 cell model, is depicted in Fig.1 as cumulative transport over time. Isoflavones permeation through Caco-2 cell monolayer increased over time for all isoflavones. After 120 minutes, the basolateral recoveries for each isoflavone in the different supplements were for daidzein: 5.80 ± 0.48% (S1); 7.15 ± 0.43% (S6); 5.47 ± 0.53% (S11); genistein: 2.70 ± 0.37% (S1); 4.75 ± 0.43% (S6), 8.93 ± 0.46% (S11); formononetin: 27.8 ± 1.52%(S11); and for biochanin A 27.9 ± 2.32 % (S11). Table 5 summarizes the isoflavones apparent P*app* in the Caco-2 model. In the red clover supplement (S11), the permeability of formononetin was similar to that of biochanin A, a similar observation was made by Wang, Chen, Joseph and Hu (2008), when studying red clover food supplements, which is in agreement with the robustness of the Caco-2 model. Interestingly, in the three analysed supplements, the P*app* for genistein was lower than for genistein as pure compound (control). This may be

due to the complexity of the extract compared to the pure solution, as the extracted matrix also possess additional components responsible for the diffusion saturation, resulting in the delay of permeability kinetics for genistein in the Caco-2 cells. Moreover, the commercial products also contain mucilage on their composition, which may act as a physical barrier against the free diffusion of compounds through the epithelia layer, as also observed by Jia, Chen, Lin and Hu (2004). This may also justify the P*app* differences between different extracts.

4. Conclusions

The proposed method to quantify isoflavones in food supplements is based on a MSPD extraction in the presence of an internal standard, followed by the direct HPLC/DAD analysis. As far as we know, this is the first time that MSDP is applied to isoflavones quantification in food supplements. The results obtained show significant differences between labelled and determined contents for the majority of supplements. The quantification of isoflavones in food supplements is important for the control of their contents, since they are choose by their therapeutic properties. Moreover, the apparent permeability coefficients (P*app*) of the isoflavonoids across the Caco-2 cell monolayers were found to be affected by the isoflavone concentration and the product matrix.

Reliable labelling information, better standardization, improved manufacturing practices and regulation of the market is required to assure consumers of the quality of isoflavone supplements. This study stresses the need for careful selection of isoflavone-containing food supplements by consumers, retailers and health care professionals.

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3. CONSIDERAÇÕES FINAIS

3.1 Considerações Finais

O controlo dos ingredientes dos suplementos alimentares está longe de ser assegurado por todos os fabricantes. A falta de conformidade com os valores rotulados tem sido reportada para vários produtos disponíveis no mercado, pondo em causa a função para que estão destinados. Tendo em conta o seu consumo generalizado, torna-se necessário conhecer os produtos que se encontram no mercado e os seus ingredientes, garantir a segurança das dosagens recomendadas e avaliar a real biodisponibilidade dos diversos compostos.

A regulamentação dos suplementos alimentares tem sido alvo de harmonização na UE a fim de melhorar a qualidade destes produtos e garantir a segurança dos consumidores. Têm sido publicadas listas positivas para vitaminas e minerais, relativamente às formas químicas e limites permitidos. O número de outras substâncias com efeito nutricional ou fisiológico que os suplementos alimentares podem conter está estimado em mais de 400, atualmente, no mercado europeu. Estas substâncias são regidas pela legislação nacional de cada Estado-Membro. O número avassalador de substâncias permitidas torna a tarefa da sua regulamentação e controlo um enorme desafio.

O presente trabalho teve por objetivo melhorar o conhecimento sobre diferentes grupos de suplementos alimentares disponíveis em Portugal. Foram avaliados suplementos antioxidantes, suplementos contendo ácidos gordos e suplementos à base de isoflavonas.

Tendo em conta a diversidade de compostos bioativos avaliados foi necessário recorrer a diferentes metodologias analíticas, as mais adequadas à quantificação dos compostos químicos de interesse nas diferentes matrizes.

Atualmente encontram-se no mercado diversos produtos que reivindicam propriedades antioxidantes. No entanto, a informação rotulada é geralmente escassa. Os suplementos antioxidantes presentes no mercado apresentam uma composição extremamente variada, incluindo diferentes moléculas hidrofílicas e lipofílicas, extratos naturais ou compostos sintéticos com propriedades antioxidantes. Foi determinada a atividade antioxidante dos suplementos, individualmente ou combinados, através de três ensaios *in vitro*: a atividade captadora de radicais livres 1,1-difenil-2-picril-hidrazilo (DPPH), o poder redutor, e a inibição da peroxidação lipídica na presença de substâncias reativas do ácido tiobarbitúrico (TBARS). Posteriormente, utilizou-se uma análise

discriminante linear (LDA) para categorizar as diferentes formulações e amostras combinadas de acordo com a sua atividade antioxidante. Como esperado, os resultados referentes à atividade antioxidante dos diferentes suplementos apresentaram uma grande variabilidade, o que reflete a sua diversificada composição e concentração. Em geral, os resultados obtidos confirmam o potencial antioxidante dos suplementos avaliados. Constatou-se que o consumo de misturas de antioxidantes pode oferecer algumas vantagens adicionais.

Num segundo trabalho, alargou-se a base de amostras analisadas, incluindo-se, para além de suplementos alimentares, chá verde e outras infusões de ervas e sumos de frutas, disponíveis no mercado Português. Foram analisados os compostos fenólicos totais, flavonoides e ácido ascórbico, e determinada a atividade antioxidante das várias amostras. De uma forma geral, as bebidas contendo chá verde e hibisco mostraram maiores teores de compostos fenólicos (incluindo flavonoides) e atividade antioxidante do que aquelas sem esses ingredientes. A infusão de borututu apresentou as menores concentrações de compostos bioativos e atividade antioxidante, devido à baixa quantidade de planta recomendada para preparar a bebida. Alguns sumos sem reivindicações antioxidantes no rótulo apresentaram valores semelhantes aos que apresentavam essas reivindicações. Finalmente, foram avaliados suplementos alimentares à base de selénio, utlizado pelas suas propriedades antioxidantes e anticarcinogénicas. Os resultados obtidos estavam concordantes com os níveis médios referenciados nos rótulos dos produtos.

Os suplementos alimentares ricos em ácidos gordos ómega-3 são, atualmente, dos mais populares no mercado. Os níveis de óleo, EPA, DHA, ALA e vitamina E foram determinados e comparados com os valores rotulados. Verificaram-se diferenças significativas entre os resultados experimentais e a informação apresentada no rótulo na maioria das amostras estudadas.

Os suplementos à base de plantas contendo fitoestrogénios são procurados por muitas mulheres para atenuar a sintomatologia associada à menopausa. Para a determinação do perfil de isoflavonas destes produtos foi otimizado e validado um método simples de MSPD-RP-HPLC-DAD. O método proposto foi aplicado com sucesso na análise de nove agliconas e glicosídeos. A maioria das amostras apresentou diferenças significativas relativamente aos teores de isoflavonas rotulados. A biodisponibilidade de três suplementos alimentares (isoflavonóides de soja padronizados, extrato de soja e trevo vermelho) foi investigada usando a linha celular Caco-2. A biodisponibilidade das isoflavonas presentes nos extratos parece depender da concentração de isoflavona presente e da matriz do produto.

Estes resultados sublinham a necessidade de uma melhor padronização dos compostos utilizados na preparação dos diversos suplementos, de forma a ser assegurado que fornecem efetivamente as dosagens rotuladas, a fim de tirar o maior benefício dos compostos bioativos presentes.

Atualmente, o controlo da qualidade e padronização destes produtos, a determinação da real biodisponibilidade dos diversos compostos, e a avaliação dos efeitos destes compostos bioativos na saúde, constituem um enorme desafio. Juntando ainda as questões da segurança e da toxicidade, a estabilidade do produto e a possibilidade de adulteração, torna-se evidente que há um importante trabalho a fazer para melhorar a compreensão dos diferentes compostos utilizados na preparação de suplementos alimentares.

Procurando a melhoria do sector, é necessário que todos os intervenientes relevantes (produtores, autoridades reguladoras, técnicos de saúde e investigadores) trabalhem em estreita colaboração para fornecer soluções para a gestão correta destes produtos, com o objetivo de oferecer produtos de elevada qualidade e procurando melhorar a informação disponível para que o consumidor possa ser devidamente informado dos riscos e benefícios do seu consumo.